DOCTOR OF PHILOSOPHY

Identification and molecular characterisation of elicitor-priming for resistance in tomato against Botrytis cinerea

De Vega Perez, Daniel

Award date:
2018

Identification and molecular characterisation of elicitor-priming for resistance in tomato against *Botrytis cinerea*

Daniel de Vega Pérez

Doctor of Philosophy

School of Life Sciences, University of Dundee

January 2018

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List of Abbreviations

ABA	Abscisic acid
ACC	1-aminocyclopropane-1-carboxylic acid
ACIK1	Avr9/Cf-9 induced kinase 1
ACRE	Avr9/Cf-9 Rapidly Elicited
ACRE75, 180	Avr9/Cf-9 Rapidly Elicited 75, 180
AP2	APETALA2
AOS	Allene oxide synthase *(*AOS*)*
ATMT	*Agrobacterium tumefaciens*-mediated transformation
Avr	Avirulence
BABA	β-aminobutyric acid
BAK1	Brassinosteroid insensitive 1-associated receptor kinase 1
bHLH	Basic helix–loop–helix (bHLH)
BLAST	Basic Local Alignment Search Tool
BR	Brassinosteroid
BTH	Benzothiadiazole
CaBP	Calcium-binding protein
CAM	Calmodulin
Cas9	CRISPR associated protein 9
cDNA	Coding DNA
CDS	Coding sequence
CEBiP	Chitin elicitor-binding protein
CHI	Chitinase
Cf-2/Cf-4/ Cf-9	*Cladosporium fulvum*-2/4/9
CMPG1	Cys, Met, Pro, and Gly protein 1
Col-0	Columbia-0
CRISPR	Clustered Regularly Interspaced Short Palindromic Repeats

Cv	Cultivar
DA	Deacetylation degree
DAMPs	Damage-associated molecular patterns
DAT	Days after treatment
ddH_2O	Double-distilled water
DMI	Demethylation inhibitor
DNA	Deoxyribonucleic acid
DPI	Days post inoculation
DSB	Double-Strand DNA Break
DTT	DiThioThreitol
EB	Elution buffer
EDTA	Ethylene Diamine Triacetic Acid
EF1-α	Elongation factor 1 alpha
eGFP	Enhanced GFP
ERF	Ethylene Responsive Factor
ET	Ethylene
ETI	Effector-triggered immunity
ETS	Effector-triggered susceptibility
EV	Empty vector
FGSC	Fungal Genetics Stock Center
FLARE	Flagellin Rapidly Elicited genes
Flg22	22 amino acid motif from bacterial Flagellin
FLS2/3	Flagellin sensing 2/3
GC	Germinated conidia
gDNA	genomic DNA
GFP	Green Fluorescent Protein
HG	Homogalacturonan
HPLC	High Performance Liquid Chromatography

HR	Hypersensitive Response
IBI1	IMPAIRED IN BABA-INDUCED IMMUNITY 1
IPTG	β-D-1-thiogalacto- pyranoside
IR	Induced Resistance
ISR	Induced systemic resistance
JA	Jasmonic acid
JA-Ile	Jasmonoyl- L- isoleucine
LapA	Leucine aminopeptidase A
LB	Luria-Bertani
LoxD	Lipoxygenase D
LPS	Lipopolysaccharides
LRR	Leucine-rich repeat
LRR-RLKs	Leucine-rich repeat receptor-like protein kinases
LRR-RLPs	Leucine-rich-repeat receptor protein
MAMP	Microbe associated molecular pattern
MAPK	Mitogen-activated protein kinase
MeJA	Methyl jasmonate
MMP	Matrix metalloproteinases
mRFP	monomeric RFP
mRNA	messenger RNA
MS/MS	tandem mass spectrometry
NB-LRR	Nucleotide Binding and Leucine-Rich Repeat
NCBI	National Center for Biotechnology Information
NIP	Necrosis-inducing proteins
NLP	Necrosis and ethylene-inducing peptide 1-like protein
NPR1	Nonexpressor of Pathogenesis-Related protein1
OG	Oligogalacturonide
ORA59	OCTA- DECANOID-RESPONSIVE ARABIDOPSIS AP2/ERF 59

PAMP	Pathogen associated molecular pattern
PBS	Phosphate-buffered saline
PCD	Programmed cell death
PCR	Polymerase chain reaction
PDA	Potato Dextrose Agar
PDB	Potato Dextrose Broth
PDF1.2	PLANT DEFENSIN 1.2
PG	Polygalacturonase
PGN	Peptidoglycan
PGPR	Plant growth-promoting rhizobacteria
Pin/PI-1, 2	Proteinase inhibitor-1, 2
POD	Peroxidase
PR	Pathogenesis-related
PR-1	Pathogenesis-related gene 1
PRR	Pattern recognition receptor
PTI	PAMP triggered immunity
PTI5	Pathogenesis-related genes transcriptional activator
qRT-PCR	Quantitative Reverse-Transcriptase PCR
QTL	Quantitative trait loci
UPL	Universal Probe Library
R	Resistance
RBOHB	Respiratory burst oxidase homolog protein B
RFP	Red Fluorescent Protein
RLK	Receptor-like kinase
RLP	Receptor-like protein
RNA	Ribonucleic Acid
RNAi	RNA interference
ROS	Reactive oxygen species

RT	Room temperature
RT-PCR	Reverse transcription polymerase chain reaction
SA	Salicylic Acid
SAR	Systemic-acquired resistance
SD	Standard deviation
SEM	Standard error of the mean
SDS	Sodium dodecyl sulfate
SDW	Sterile distilled water
SP	Signal peptide
SYBR	Synergy Brands, Inc.
TFs	Transcription factors
UPRT	uracil phosphoribosyltransferase
X-gal	5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside

Ackowledgements

This journey finally comes to an end. It has been four long years. First, I would like to thank my supervisor, Adrian, and to my co-supervisor Nicola, for choosing me to do this great and beautiful Ph.D project, for all your support, pacience and advice throughout all this time. It was a great privilege to be part of the James Hutton Institute team. Also I am really grateful to my amazing lab team, Sonia, Emma and Lauren, thank you so much for welcoming me to your lab team and for your help and support with my experiments these 4 years, you are in my heart. Also thanks a lot to Louise, Ashleigh and Jackie for all your help. I also want to thank Pete Hedley for your great help with the Microarray, the BioSS department, especially to Colin, for your knowledge and stats teachings, Hazel for your help with my cloning and Julie for your scientific knowledge and support; the glasshouse team: Jim, Aly, Graham, Sarah, Alison, Loeis. Special thanks to my friends, Lucie, for all our days and nights discussing about our experiments and giving support to each other; to Carmen, for all your kindness and great help; to Fulvia, GianLuca, Nora, Adeline, Gaetan and Camille my great friends all these years. To my favourite yogis Rapha and Estela. To my Sheffield friends Edu and Maria.

A mi madre, Mercedes, por todo tu amor, apoyo y cariño incondicionales; por todas nuestras profundas conversaciones, tu paciencia escuchándome y tu gran inteligencia emocional, que me han dado ánimos cuando más los necesitaba, por tu tremenda energía y fuerza vitales que me has enviado, me han ayudado a iluminar mi camino en los momentos de zozobra. A mi padre, Félix, por toda tu sabiduría y tu alegría contagiosas, por todos los valores que me has inculcado, sobre la determinación, la disciplina, el Poder del Ahora y de mi Verdadero Yo, tu maravillosa risa a carcajadas y tu punto de vista sobre la vida, me han guiado entre las distracciones y la bruma más intensas a visualizar mi meta con claridad. A ti Gonçalo, por haberme escogido para crecer juntos estos últimos tres años, sin tu apoyo, amor y bondad infinitas el camino no hubiese sido lo mismo. A ti Estrella, por decidir ser mi mentora, por tu esfuerzo, dedicación sin parangón, nuestras charlas científicas y amistad incondicionales, te estaré siempre agradecido. A vosotros cuatro, los pilares y estrellas polares de mi travesía que me han sostenido y guiado a lo largo de estos años, os dedico mi Tesis Doctoral y os guardo un pedazito de mi Corazón…

Declarations

The results presented in this Ph.D thesis are from investigations conducted by myself. Any work that is not my own is clearly identified with the appropriate references and publications.
I hereby declare that I am the author of this work and it has not been previously accepted for any higher degree.

Daniel de Vega Pérez

We certify that Daniel de Vega Pérez has fulfilled the relevant ordinance and regulations of the University Court and is qualified to submit this thesis for the degree of Doctor of Philosophy.

Professor Adrian Newton, The James Hutton Institute

Abstract

Conventional crop protectants (fungicides) can lose their efficacy due to selection pressure for pathogen resistance caused by their widespread use[1]. To date, there is a lack of genetic resistance in commercial crop varieties against necrotrophic fungal pathogens[2], such as *Botrytis cinerea*. The aggressive fungal pathogen *Botrytis cinerea* infects almost all vegetable and fruit crops[3] (>1400 plant species), killing the host by inducing necrosis with degradation enzymes (virulence factors) and manipulating its host defences. Non-host inducing agents, such as elicitor molecules, are able to stimulate pathogen-induced defence mechanisms in the plant[4] and induce plant defences for increased and more efficient resistance (priming) against pathogens such as *B. cinerea*. Priming is based on a fine-tuned and enhanced resistance to biotic/abiotic stress that results in a faster and stronger expression of resistance upon pathogen attack[5]. This study aimed to identify candidate elicitors, determine their mode of action in the plant-*B. cinerea* interacion, characterise their molecular function and investigate a candidate elicitor role in priming tomato against *B.cinerea*. Resistance phenotypic assays have revealed that chitosan, a MAMP, was able to induced resistance in solanaceous crops *Solanum melongena*, *Nicotiana benthamiana*, *Solanum lycopersicum* and brassicaceous plant *Arabidopsis thaliana* by significantly decreasing necrotic lesion sizes and priming for callose deposition in a concentration-dependent manner. Furthermore, large-scale double (host/pathogen) transcriptomic analysis has unveiled that chitosan was able to prime 1,745 tomato transcripts during early and asymptomatic stages of *B. cinerea* infection. Transcriptome-based gene ontology (GO) enrichment and HPLC/MS analyses revealed that chitosan-priming targets five main clusters, incuding 1) a higher cell sensitization throughout a faster and stronger transmembrane receptor/receptor-like kinase, CaBP and signal transducer activity; 2) a cell-wall reinforcement through R protein activation, cellulose synthesis and PGs, PMEs and xyloglucan repression; 3) a fine-tuned potentiation of JA/JA-Ile synthesis and JA/ET/SA/ABA transcriptional regulation; 4) an induction of the lipid/fatty acid metabolism and phenylpropanoid pathway; and 5) a strong repression of *B. cinerea* PGs, *BcSOD*, hexokinase and novel virulence factor uracil phosphoribosyltransferase (*BcUPRT*). Transcriptome analysis helped to the identification of two tomato novel and co-expressed genes, *SlACRE75* and *SlACRE180*. Both transcripts and their *N. benthamiana* homologs were primed by chitosan early during infection and encode small proteins without a signal peptide and with unknown functions. Subcellular localization indicates that the four proteins are involved in intracellular/cytoplasmatic signalling. Finally, transient and constitutive overexpression of *SlACRE75*, *SlACRE180* and their *N. benthamiana* homologs revealed that they are positive regulators of plant resistance against *B. cinerea*. Identification of specific chitosan-primed tomato pathways and genes such as *ACRE75* and *ACRE180*; and *BcUPRT*, will provide a

valuable resource for developing novel fungicide use strategies and engineering non-host resistance against necrotrophs in dicots.

References: [1]Pappas, 1997; [2]Smith et al. 2014; [3]Weiberg et al. 2013; [4]Aranega-Bou et al. 2014; [5]Conrath, 2011

1 Chapter 1. General Introduction

1.1 Population growth: food production increase needed

Human population has increased exponentially in recent decades reaching 7.6 billion in 2017 and projected to reach 8.6 billion by 2030 (United Nations, The 2017 Revision of World Population Prospects). The challenge to feed more than 9 billion people by 2050 which will increase the global demand for food availability, stability, access, and utilization, also termed food security (FAO), which will have a big impact on agriculture productivity requirement. Thus, in order to avoid hunger worldwide, humanity will have to focus on some key factors in order to improve food security and to enhance agricultural production, such as, abiotic stresses, which can cause up to an estimated crop yield loss of 70% (Banerjee & Roychoudhury 2015), climate change, which has complex impacts on food security and agriculture (Schmidhuber & Tubiello 2007), land use, water and energy consumption, land urbanization and pests and diseases challenges (Gregory et al. 2009; Charles et al. 2012; Schmidhuber & Tubiello 2007).

1.2 Crop economical losses due to pest and pathogens and conventional crop protection

Crop yield loss due to pests and pathogens is one the great challenges that has affected mankind since the beginnings of agriculture. Mankind has dealt with pests and diseases and managed to learn how to control them over the years, however pathogens evolve rapidly and manage to overcome control efforts. Moreover, crop yield losses due to pests and diseases are responsible for a decrease of 20-40% of agriculture productivity (Savary et al. 2012; Oerke 2006). Quantification and analysis of the total impacts of pests and diseases on crop systems worldwide is a complex matter that challenges scientists and requires application of models understand the key drivers (Donatelli et al. 2017). Pre-harvest damage alone by pests and pathogens in eight important food and cash crops is valued at US$300 billion (Anderson et al. 2004).

A major challenge in the fight against pathogen attack to crops worldwide is the ineffectiveness of conventional crop protectants due to pathogen resistance. Conventional crop protectants (e.g. fungicides) can lose their efficacy due to selection pressure for pathogen resistance caused by their widespread use (Pappas 1997) or the emergence of new pathogens. This is not a new phenomenon as, for example, after fungal resistance to benzimidazoles in the 1970's, extensive use of some newer fungicides, such as dicarboximides, has subsequently led to the appearance of resistant *B. cinerea* strains (Pappas 1997). Pesticides are also limited by European regulations due to human health and environmental issues. The recent European Directives "Plant Protection Products Regulation" 1107/2009 and the "Sustainable Use Directive" 2009/128/EC are the latest in a series of legislative changes that aim to reduce pesticide use in Europe. One of the main elements of the Regulation 1107/2009, unlike the Directive, is that it provides the possibility to reject active substances on the basis of their intrinsic hazard properties rather than their risk (Figure 1.1) (Williams 2011). This is also critical for the control of bacterial pathogens, for which there are no effective alternatives.

Human Health	Environmental
Carcinogen C1A & C1B	PBT (Persistent, Bioaccumulative & Toxic)
Mutagen M1A & M1B	POP (Persistent Organic Pollutant)
Toxic for Reproduction R1A & R1B	vPvB (very Persistent, very Bioaccumulative)
Endocrine disruptor	Endocrine disruptor

Figure 1.1 Plant Protection Products Regulation 1107/2009. Criteria for the approval of active substances.

New products can be lost from the market also for regulatory reasons, and the market sometimes requires crop varieties to be grown that are susceptible. Therefore, present crop protection strategies are aimed at reducing usage of toxic active ingredients. In recent decades, research on more benign alternatives to control pathogens has become a priority. However, there are other issues that also affect fungicides, as continuous application and usage of fungicides can also be energy demanding (Luna, López, et al. 2014). One potential replacement for pesticides and fungicides can be products that induction of plant's endogenous defence mechanisms.

1.3 Types of microbes and plant-microbe interactions

Plant-microbe interaction is a complex matter than can be multifaceted. Depending on the type of interaction with their host, microbes can be divided in parasites, mutualists and pathogens, however, there is a gradient in which these interactions occur and depending on the microbe life cycle stage and environmental conditions this relationship can change (Newton et al. 2010). According to the type of host-microbe interaction, symbiotic relationships can be mutualistic, commensal or parasitic. In particular, mutualistic crop-microbial interaction is well-known to have great beneficial outputs for the agriculture (Wasternack & Hause 2013), including root nodule symbiosis (RNS), arbuscular mycorrhiza (AM) fungi and plant growth-promoting rhizobacteria (PGPR), which live in the plant rhizosphere and are well-reported to be beneficial for the plant growth and defences (Cawoy et al. 2014; Choudhary & Johri 2009; Song et al. 2015; Santos et al. 2014). Traditionally in plant pathology microbes can be divided into endophytes, which colonize plant tissue without causing disease symptoms; saprophytes, that decay plant-related material; and pathogens, which cause disease (van Kan et al. 2014). Depending on the pathogen life style, plant pathogens can be biotrophs, when they feed on living host cells and tissue and they use effector molecules to suppress host immunity, such as the fungi *Cladosporium fulvum*, *Puccinia graminis* and *Blumeria graminis* f.sp. *tritici* (*Bgt*), which causes powdery mildew disease and it is an important source of crop yield loss worldwide being able to infect monocotyledonous and dicotyledonous plants (Tayeh et al. 2013); necrotrophs, when their main strategy is to kill their host cells and tissue and feed on the nutrients, such as *Botrytis cinerea*, *Sclerotinia sclerotium*, *Fusarium oxysporum*, *Phaeosphaeria nodorum*, *Alternaria brassicicola* and *Alternaria solani*; and hemi-biotrophs, when they go through a biotrophic stage and subsequently switch to a necrotrophic phase, a classical example of which is the oomycete *Phytophthora* spp., which are a class of filamentous and destructive plant-pathogens (Jupe et al. 2013) and the model plant bacterial pathogen *Pseudomonas syringae* that infects a wide range of plants and causes important economic losses worldwide (Xin & He 2013).

Necrotrophic fungi are the largest class of fungal phytopathogens that pose an economic threat to crops worldwide (Łaźniewska et al. 2010), surpassing biotroph-related losses, for example in Australia where the necrotrophs *Pyrenophora tritici-repentis* and

Stagonospora nodorum on wheat and barley exceed losses by rusts and mildews (Murray & Brennan 2010).

Fungal necrotrophs can be categorized into host-specific, which can produce host-specific toxins (HSTs) such as *Cochliobolus carbonum*, *Phaeosphaeria nodorum* and *P. tritici-repentis,* and broad-host range, such as *B. cinerea*, *A. brassicicola*, *Plectosphaerella cucumerina*, and *S. sclerotiorum* (Wang et al. 2014).

1.4 The pathogen: *Botrytis cinerea*

1.4.1 Biology and life cycle

Botrytis cinerea Pers. Fr. *(teleomorph* (teleomorph *Botryotinia fuckeliana*) causal agent of grey mould disease in tomato and other crops, is a filamentous fungus that belongs to the phylum of *Ascomycota*. It is characterized by abundant asexual tree-branch-like and grey structures called conidia. It also produces highly resistant structures called *sclerotia*, which are compact masses of tough fungal mycelium containing food reserves. One role of *sclerotia* is to overwintering environmentally extreme conditions such as cold and/or dry (Figure 1.2). *B. cinerea* is an airborne opportunistic pathogen with a broad-host range, it is notoriously aggressive on fleshy fruit (Cantu et al. 2009) and it spreads its spores either through the air movement or rain splash.

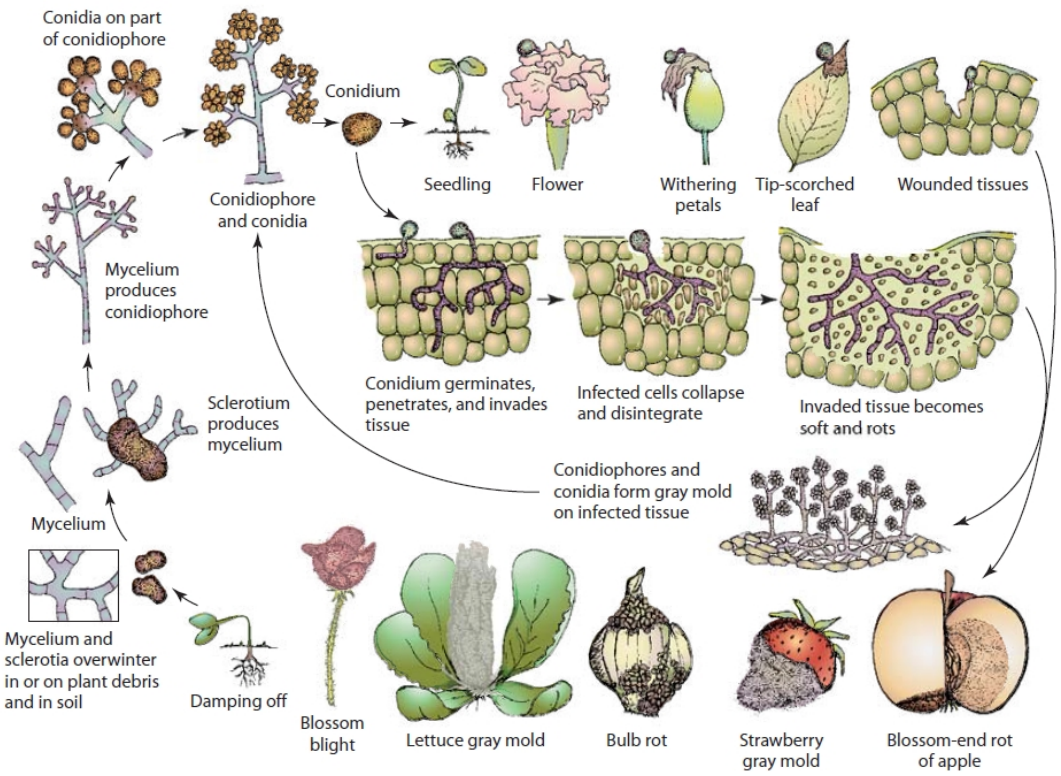


Figure 1.2. Disease cycle of *Botrytis cinerea* (Agrios, 2005).

B. cinerea conidia are released from hyphae and blown onto leaf surfaces. Once conidia have landed on the surface of a susceptible host (Figure 1.2), there are several factors that can affect the asexual spore germination. Free surface water or high relative humidity (>93% RH) is essential to germinate and penetrate the host epidermis (Williamson et al. 2007). It also requires cool weather (18–23°C) for best growth, sporulation, spore release, germination, and establishment of infection. During active growth, it produces a range of hydrolytic enzymes and metabolites to facilitate penetration and colonization of host tissues (Kars et al. 2005). Conidia form a grey mould on infected tissue and subsequently, infected cells collapse and disintegrate (Figure 1.2).

1.4.2 *Botrytis cinerea* pathogenicity

B. cinerea is a fungal generalist (broad-host range) considered to be a model necrotrophic pathogen (Williamson et al. 2007), causative of grey mould disease in tomato and other economically important crops and softfruits, such as pepper, aubergine, grape, lettuce and raspberry, that causes annual losses between $10-$100 billion. *B. cinerea* can cause disease in crops from the Solanaceae family, trees and

ornamental plants (Williamson et al.2007; Staats et al. 2005; Muñoz & Moret 2010) and it can cause spots, rot and blight in the field (post-harvest) as well as in greenhouses (Finkers et al. 2007). Thus, *B. cinerea* can infect and cause disease on almost all vegetable and fruit crops (Weiberg et al. 2013), at least 235 host species (Choquer et al. 2007), most of which are dicotyledonous, including important plant species that are used for food, oil, protein and fibre extraction. Some monocotyledonous plants are also susceptible to attack by a group of related *B. cinerea* species specialized to infect about a dozen such hosts (Williamson et al. 2007). Product quality of horticultural crops has been the main area of research in recent decades. Growers and sellers have been looking for best possible product quality and maximum profits (Darras 2011). *B. cinerea* is able to reduce the yield of the crop before it is harvested (pre-harvest yield loss) causing massive losses in greenhouses. Its aggressive nature makes it very destructive in mature or senescent tissues of crops and fruits, which strongly reduces the quality of the product, making it one of the major causes of post-harvest waste and spoilage worldwide. However, there is evidence that *B. cinerea* can be an opportunistic pathogen and infect at a much earlier phase of the plant development, being able to colonize unripe plant tissue and remain in a quiescent and/or asymptomatic state (Figure 1.3) (Van Kan 2000; Benito et al. 1998), presumably waiting for favourable environmental conditions to start spore germination and rot host tissues. Furthermore, it has been reported that *B. cinerea* can infect some plants, such as *Primula x polyantha*, and grow systemically without any symptoms, as an endophytic infection (Barnes & Shaw 2002). Experiments have also showed that *B. cinerea* is often present in symptomless lettuce plants as a systemic endophytic infection which may arise from seed (Sowley et al. 2009).

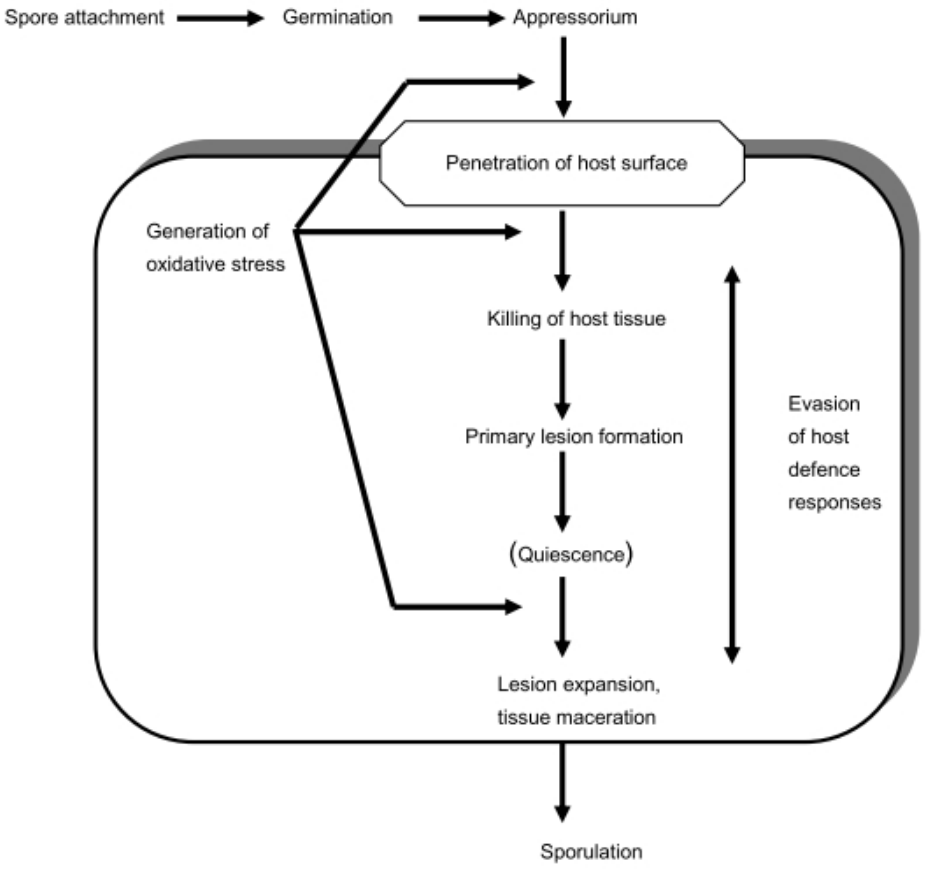


Figure 1.3 Model of the different stages in the infection process of *Botrytis cinerea*. The shaded box represents the host tissue (Van Kan 2000).

1.5 Issues to control *Botrytis cinerea*

To date, there is a lack of genetic resistance in commercial crop cultivars against fungal aggressive pathogens (Smith et al. 2014; Williamson et al. 2007) such as *B. cinerea*, *A. Solani* and *S. sclerotium*. *B. cinerea* is considered the second most important fungal pathogen worldwide, right after rice blast disease fungus *Magnaporthe oryzae*, based on its economic and scientific importance (Dean et al. 2012). Moreover, although *B. cinerea* infection strategy is complex and not fully understood, J. A. L. Van Kan, 2005 showed that *B. cinerea* is an opportunistic and versatile pathogen that is able to infect all parts of the plant (stem, leaves, flowers and fruits), it can initiate infection on wounded plant tissue, sites previously infected by other pathogens and even healthy or undamaged tissue by directly by penetration of the cuticle (van Kan et al. 1997).

B. cinerea is also able to alter reactive oxygen species (ROS) and to manipulate its host defences by releasing lysis and necrosis-inducing enzymes (El Oirdi et al. 2011; Finiti

et al. 2014; van Kan 2006). Moreover, the *B. cinerea* multifaceted infection strategy is to date is poorly understood, this includes a recently revealed early secretome (Espino et al. 2010) that includes an array of necrosis-inducing proteins, active oxygen species (AOS)-altering proteins, a large number of cell-wall pectin and cellulose proteases and numerous proteins with unknown function. In particular, in order to manipulate its host defences, *B. cinerea* possesses genes that are able induce pathogenicity (termed 'virulence factors'), some of them strain-specific and others conserved with other fungal pathogens, such as BcNEP2, xylanases like Xyn11A, endopolygalacturonase genes such as B*cpg*1, glucoamylases like BcGs1 (Choquer et al. 2007; Ten Have et al. 1998). Furthermore, it was recently discovered that *B. cinerea* has new potential virulence factors involved in germination (Gonzalez-Rodriguez et al. 2014). *B. cinerea* also has effector-type molecules including a BcNEP1-like protein, that induces necrosis in the host, and the recently found small RNAs (Bc-sRNAs) that are able to silence *A. thaliana* and *S. lycopersicum* immunity-related genes (Weiberg et al. 2013; Wang et al. 2014; Schouten et al. 2008).

Thus, the *B. cinerea* complex represents a versatile but still unknown infection strategy, together with its ability to develop resistance towards old fashion and newer fungicides such as benzimidazoles and dicarboximides (Pappas 1997) and the fungicide limitations by European Regulations due to human health and environmental issues previously mentioned, makes it a highly difficult pathogen to control. For these reasons, a single control strategy to cope with this pathogen is both ineffective and inappropriate and a new understanding of the pathogen microbial competitors, its microenvironment and interaction with the host is needed (Williamson et al. 2007). Therefore, novel crop protection strategies need to be implemented in order to fight against this fungal aggressive pathogen. This Ph.D project was designed to investigate a novel alternative and useful strategy in crop protection within an Integrated Pests and Disease Management (IPM) framework.

1.6 Plant immune system

Plants are sessile organisms with a remarkable and sophisticated immune system that confers protection against a large number of microbes and abiotic stress (Motion et al. 2015). The exploitation of this immune system is best implemented in the context of Integrated Pests and Disease Management (IPM). Unlike for pests, there is not an easy biocontrol approach for fungal diseases, thus we need to devise other strategies. Appropriately, even susceptible plants have inducible defence mechanisms that, if triggered in a focussed, specifically-targeted way, can prevent disease and reduce the need for conventional fungicide use.

1.6.1 Innate immunity: basal resistance

Unlike animal cells, plants depend on their innate immunity due to their lack of somatic adaptive defences (Jones & Dangl 2006). However, plants are not unprotected against the pathogens and pests that attack them. They have a developed and sophisticated immune system that must be able to endure attacks from a wide variety of microorganisms, such as bacteria, oomycetes, fungi and viruses. Despite the fact that every pathogen has a different host range depending of its nature and specialization level; it is well-known that pathogens have coevolved with plants over millions of years (Roberts 2013; Stukenbrock & McDonald 2009) to develop a way to infect them, at the same time that plants have also developed more or less successful ways to resist pathogen infection and disease development. This coevolutionary development of the plant immune system has recently been accepted and represented by a zig-zag model (Figure 1.4) (Jones & Dangl 2006; Hein et al. 2009).

Many pathogens, such as oomycetes, aphids and fungi are able to penetrate directly their host cell wall, unlike plant viruses and bacteria, which depend on natural openings or vectors (Ahmad et al. 2010). In order to fight pathogen infection, plants have created a series of resistance mechanisms. As a first physical defence, plants have a waxy layer on their leaf surfaces beneath which are a series of cell-wall defences, such as lignin and callose appositions, so-called papillae. If a pathogen attempts to infect and subsequently cause disease in the plant, firstly it needs to overcome these physical barriers. This callose-rich papillae deposition is usually induced ubiquitously in the plants, in comparison with other type of defence pathways, upon pathogen attack (Voigt 2014).

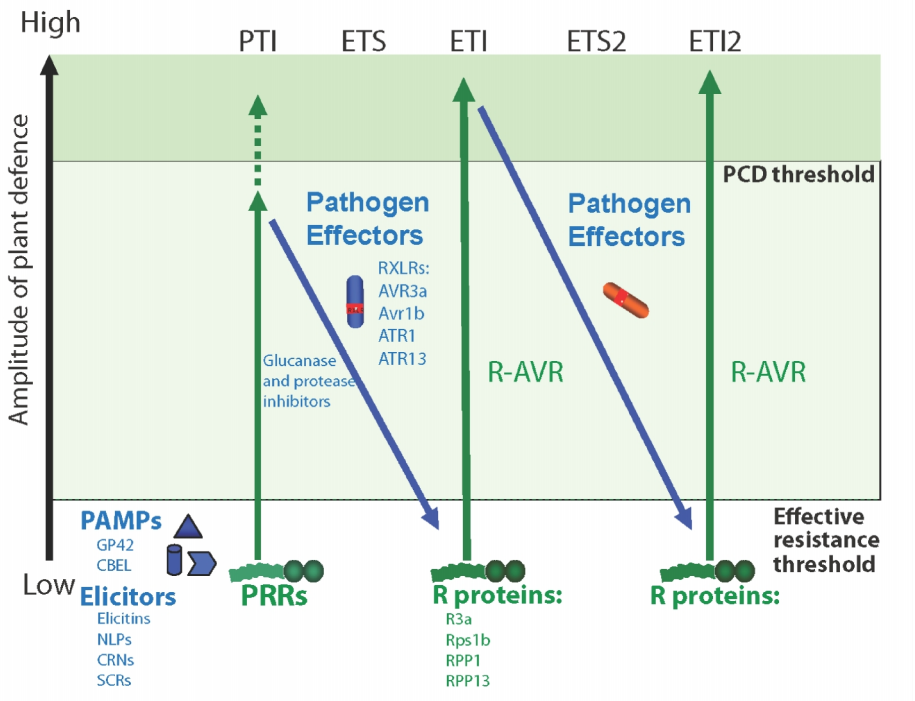


Figure 1.4 The zig-zag model in oomycete–plant interactions (Hein et al. 2009) (modified from Jones and Dangl, 2006). Shown are oomycete and other type of elicitors that are pathogen-associated molecular patterns (PAMPs) and trigger PAMP-triggered immunity (PTI) and necrosis [dotted arrow extending PTI beyond the threshold for host programmed cell death (PCD)]; examples of oomycete effectors that contribute to effector triggered susceptibility (ETS); and examples of host resistance proteins that detect oomycete effectors to trigger immunity (ETI). The amplitude of defence is shown on the y axis, and the threshold for activation of host PCD is also indicated. CBEL, cellulose-binding elicitor lectin; CRN, crinkling and necrosis; NLP, Nep1-like protein; PRR, pattern recognition receptor; R, resistance; SCR, small cysteine-rich.

If a pathogen does manage to penetrate through these layers, the plant needs to be able to combat it (Figure 1.4). As a primary defence response, plants have a wide range of specific cell-wall surface receptor-type proteins called pattern-recognition receptors (PRRs) that respond to microbes through the sensitive and quick recognition of conserved microbial features (Zipfel 2014), such as chitin, flagella, glycoproteins or lipopolysaccharides, called microbe-associated molecular patterns (MAMPs) and pathogen-associated molecular patterns (PAMPs), or molecules released on damaged tissue called damage-associated molecular patters (DAMPs) (Ahmad et al. 2010). This recognition triggers a set of defence mechanisms in the plant that results in the activation of PAMP-triggered immunity (PTI) which can prevent the pathogen from infecting and colonising host tissues.

It has been discovered that successful pathogens have acquired specific host-specific molecules called effectors, such as avirulence (Avr) proteins and small RNAs (Weiberg et al. 2013), that they release to prevent host recognition of their PAMPs/MAMPs or by directly suppressing PTI responses (Bardoel et al. 2011). In a constant plant-pathogen arms race, plants acquired a second layer of immune response in which they can recognise effectors with resistance (R) proteins and subsequently trigger so-called effector-triggered- immunity (ETI) (Pieterse et al. 2012). This coevolution between the pathogen and the host, where the pathogen avirulence (Avr) gene evolves to avoid recognition and the host resistance (R) gene changes in order to scan and recognize pathogen MAMPS/PAMPS is accepted as the distinctive avirulence (Avr) gene-for-gene model (Stukenbrock & McDonald 2009).

During this plant-pathogen interaction there is an onset of defence systems tiggered by the plant which leads to resistance or, in the worst case, disease development. The concept of plant disease resistance and the gene-for-gene interaction model were first described with the study of the flax interaction with the rust causing pathogen *Melampsora lini* (Flor 1955, 1971). The theoretical basis of this model states that for each dominant gene conferring resistance in the host, there is a corresponding dominant pathogenicity gene conferring avirulence in the parasite (Flor 1955, 1971). This important finding suggests the presence of a large number of avirulence (Avr) genes encoding proteins that can be recognized by host resistance (R) proteins triggering a set of defence responses in plants usually leading to a localized host cell death termed hypersensitive response (HR) (Dangl 1996; Dangl & Jones 2001), which is part of the effector-triggered immunity (ETI). Alteration in any of these genes by the plant or pathogen often leads to a compatible interaction, called host susceptibility. Many different R and Avr proteins have been characterized through the years providing a better understanding of the plant-microbe interactions (Dangl & Jones 2001), including the tomato R protein Cf-4 mediating the recognition of the *C. fulvum* effector protein Avr4 (Joosten et al. 1994; Stergiopoulos & de Wit 2009), the potato R protein R3a that recognises Avr3a effector from *Phytophthora infestans* (Armstrong et al. 2005) and the recognition of AvrPto from *Pseudomonas syringae* pv tomato by receptor kinase Pto in tomato (Shan 2000).

R/Avr interaction can ultimately lead to HR production by the plant to avoid biotrophic pathogen expansion. However, plant cell necrosis can also be induced by necrotrophic pathogens, such as *B. cinerea*, to promote disease, which may be analogous to the biotroph-induced cell death during the hypersensitive response (Van Kan 2000).

1.6.2 Induced resistance: SAR, ISR, direct induction and priming

Until recently, plant defence mechanisms were explained based on basal immune responses after pathogen challenge. Basal resistance, in many cases, is not enough to survive and leads to disease and ultimately a premature death of the host. However, plants are capable of defending themselves and fight off pathogen attack through constitutive and inducible defence mechanisms (Jiang et al. 2016), resulting in a broad-spectrum and more efficient resistance, called induced resistance (IR). This way, plant resistance can be enhanced and confer greater protection against future abiotic and biotic stresses.

Induced resistance can be categorized as two different types or forms. The first form is called systemic acquired resistance (SAR) and occurs in a spatially different part of the plant from the induction point of pathogen challenge (Walters & Heil 2007). As studied in the model plant *Arabidopsis thaliana*, SAR is mainly effective against biotrophic pathogens as it depends on the activation of the salicylic acid (SA) signalling pathway (Srivastava et al. 2011; Walters et al. 2008) and requires the action of the regulatory protein NPR1 (Pieterse & Wees 2015; van Wees et al. 1999). SAR has also been associated with the systemic expression of a group of genes encoding pathogenesis-related (PR) proteins, such as PR-1, β-1,3-glucanases (PR-2), chitinases (PR-3), PR-4 and osmotin (PR-5) (Sticher et al. 1997). Unlike the gene-for-gene resistance, SAR is able to provide resistance against a broad spectrum of would-be pathogens, such as fungi, viruses, bacteria and oomycetes.

The second systemic defence is called induced systemic resistance (ISR) and is induced by certain strains of plant growth promoting rhizobacteria (PGPR) that are present in large numbers on the rhizosphere (Loon et al. 1998). Several strains of the species *Bacillus amyloliquefaciens* and *Bacillus subtilis* among others are well-characterised PGPR capable of inducing ISR in multiple crops and other plants; including tomato, pepper, muskmelon, watermelon, sugar beet, tobacco, *Arabidopsis sp*., cucumber and loblolly pine; against various viral, fungal, nematodes and bacterial pathogens

(Choudhary & Johri 2009). Unlike SAR, ISR is not associated with local necrotic formation nor with changes in the expression of PR genes and it is known that, in *A. thaliana*, ISR requires the activation of ethylene and jasmonate-dependent genes but does not depend of salicylic acid (SA) pathway (van Wees et al. 1999). Furthermore, although SAR and ISR trigger and depend on different signalling pathways, it seems to be a convergence in down-stream both pathways (Loon et al. 1998) where NPR1 plays an important role in ISR establishment (Figure 1.5) (Walters & Heil 2007).

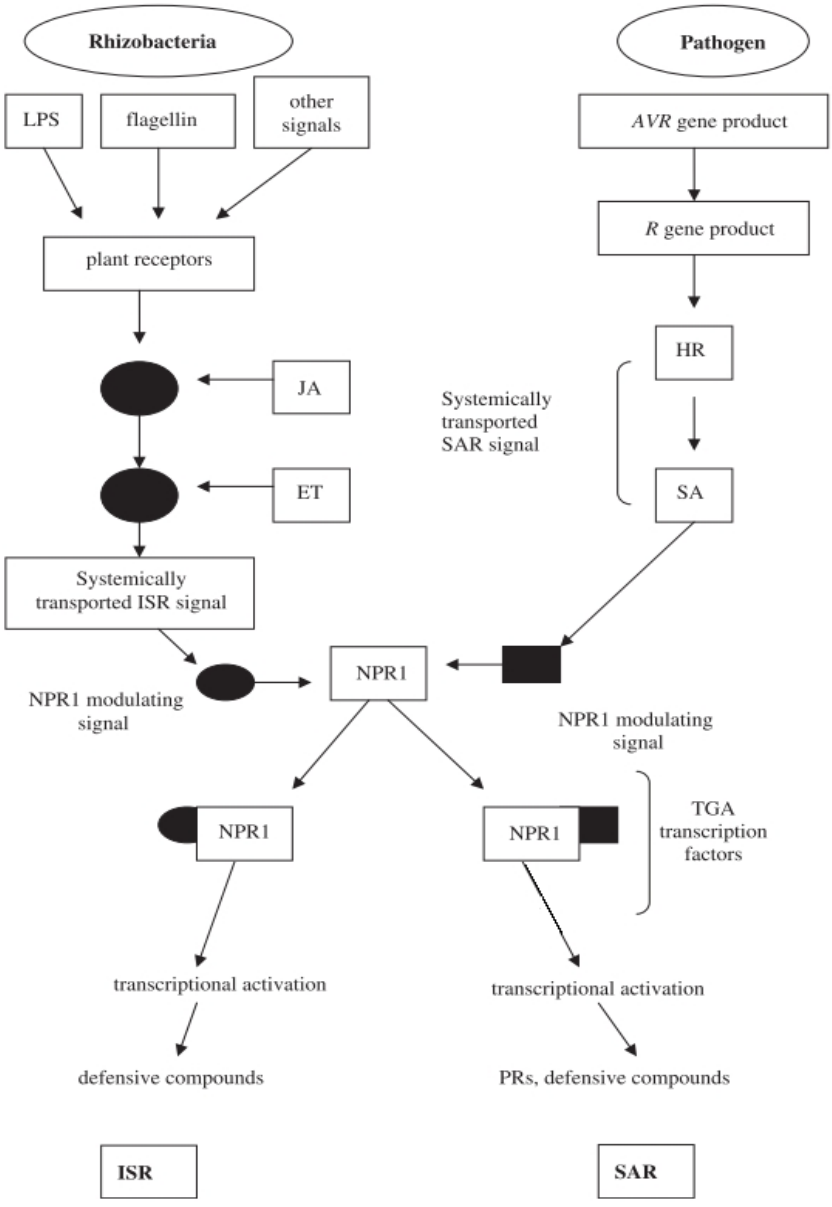


Figure 1.5 Signal transduction network controlling ISR mediated by PGPR and pathogen-induced-SAR. Adapted from (Walters & Heil 2007).

Induced resistance works under 2 different mechanisms (Aranega-Bou et al. 2014), which include a direct activation of systemic plant defences after stimuli (direct induction of defences), and a second mechanism called priming, which enables the plant to fine-tuning its defences for a more rapid and/or more robust response to abiotic and/or biotic stress (Aranega-Bou et al. 2014; Conrath 2011).

The priming process goes under 3 phases, which are a pre-priming stimulus phase or 'naïve', followed by a post-priming stimulus or 'primed state' (Figure 1.6) (Hilker et al. 2016; Mauch-mani et al. 2017) which leads into physiological, transcriptional, metabolic and epigenetic reprogramming (Luna et al. 2012), such as DNA methylation and histone modification changes; and a 'primed and triggered state' where the plant shows an enhanced resistance to pathogen challenge, mainly by a faster and/or stronger defence response (Hilker et al. 2016; Luna et al. 2012). The 'primed and triggered state' has been related to an increased, more efficient activation of the plant defence response against pathogen attack (Figure 1.6) with minimal plant fitness costs (Worrall et al. 2012; Conrath 2009). Moreover, the 'primed and triggered state' of the plant results from an amplified cell sensitization or perception (increased 'alertness') of immunity-inducing signals, rather than from the direct gene induction (Aranega-Bou et al. 2014; Slaughter et al. 2012).

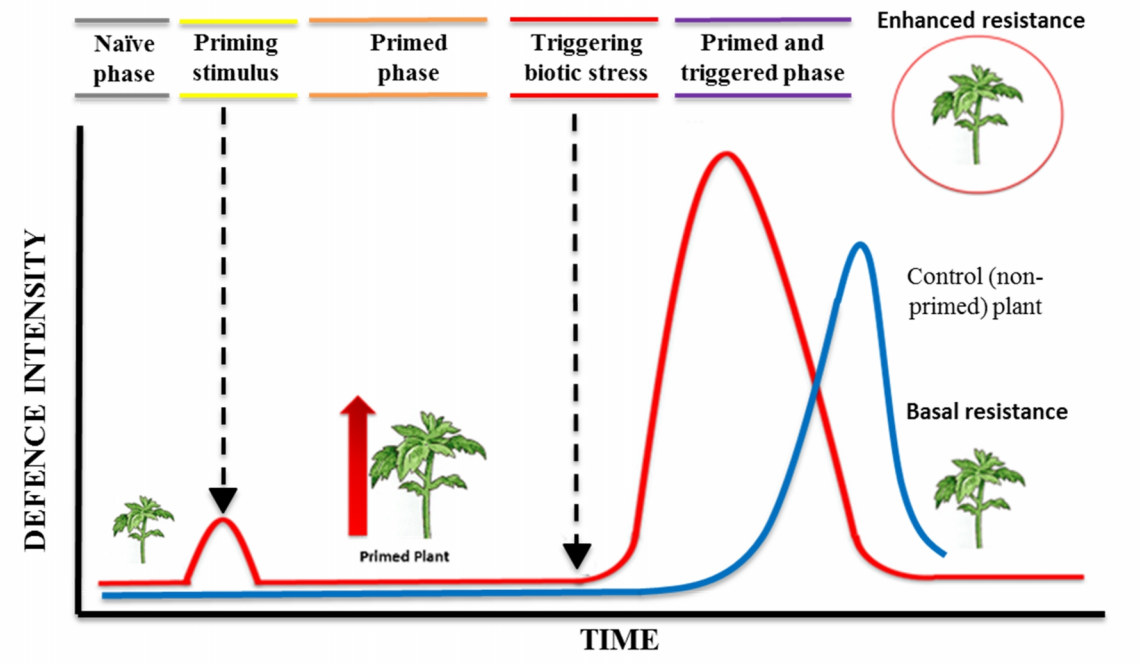


Figure 1.6 Model of a general priming process with an elicitor or priming agent. The priming stimulus (e.g. elicitor or priming agent) acts on a pre-primed or naïve organism which leads into a 'primed phase'

and precedes the stress response induced by a triggering stimulus, such as pathogen infection. After the triggering stress (e.g. pathogen attack), the 'primed and triggered' plant shows a stronger and rapid defence response which leads into and enhanced resistance against different stresses. Adapted from (Hilker et al. 2016).

1.6.3 Role of phythormones in plant immune system

Phytohormones regulate diverse aspects of plant life, such as cytokinin (CK) and auxin that are involved in root development (Gupta et al. 2013); jasmonates and ethylene which play important roles in plant development and ripening respectively (Nakata et al. 2013; Cantu et al. 2009); the hormones abscicic acid (ABA) and brassinosteroids (BR) are largely involved in plant development and growth (Aleman et al. 2016; Bajwa et al. 2013). Moreover, plant hormones play key roles in the regulation of biotic and abiotic stress responses, predominantly salicylic acid (SA), abscisic acid (ABA), ethylene (ET), jasmonic acid (JA) and its derivatives (termed jasmonates) and more recently started to study auxin, gibberellic acid (GA), cytokinin (CK) and brassinosteroids (Figure 1.7) (Kouzai et al. 2016; Leon-Reyes et al. 2009; Bari & Jones 2009). It is commonly accepted that the SA pathway is associated with immune responses to biotrophic pathogens and often antagonistic to JA, whereas JA and ET, which can act synergistically, are related to defence responses against necrotrophic pathogens and wounding by herbivorous insects (Bari & Jones 2009; Thakur & Sohal 2013; Heil & Ton 2008). Furthermore, there is evidence that these hormone-signalling pathways do not act independently and they can actually interact with each other. Indeed, cross-talk between signalling SA and JA/ET pathways, which can act antagonistically, neutrally or synergistically, plays an important role in the regulation and fine-tuning of plant induced defences that are activated after pathogen attack (Kunkel & Brooks 2002; Pieterse & Van Loon 2004; Koornneef et al. 2008; Mur et al. 2006) which may depend on the relative concentration of each hormone (Mur et al. 2006). Nevertheless, pathogens have also evolved to exploit these negative or positive pathway interactions to their benefit in order to promote disease development (El Oirdi et al. 2011), which makes priming an essential and promising tool to enhance and fine-tune plant endogenous defence mechanisms, such as hormone signalling cascades, in order to avoid or reduce pathogen manipulation.

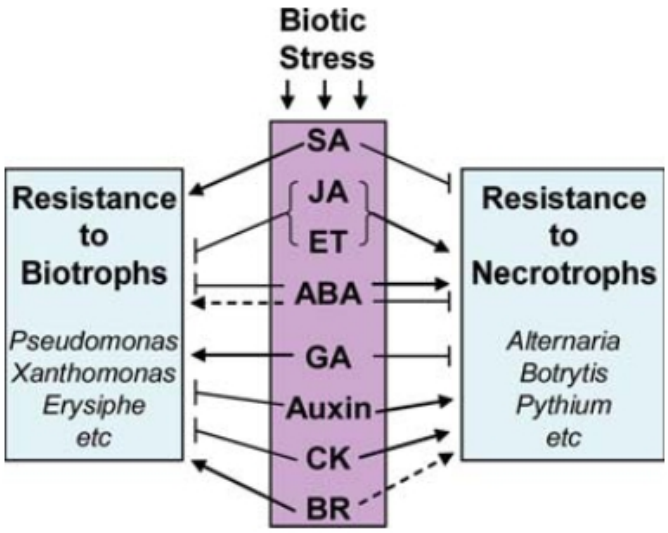


Figure 1.7 Simplified model showing the involvement of different hormones in the positive or negative regulation of plant resistance to various biotrophic and necrotrophic pathogens (Bari & Jones 2009). The arrows indicate activation or positive interaction; blocked lines indicate repression or negative interaction and dashed arrows represent hypothesized connections.

However, identification and characterisation of phytohormone role in plant defences has generally been conducted in *A. thaliana*, mainly due to the abundance of transgenic lines impaired in a gene involved in hormone biosynthesis or signalling (Derksen et al. 2013; Wiesel et al. 2015). Moreover, recent studies have acknowledged differences in the hormone responses between plant species where, despite the commonalities, signalling pathways or other regulatory systems can be unique and specific to one species (Derksen et al. 2013; Garg et al. 2012), which makes necessary species-specific hormone studies.

1.6.4 Resistance elicitors as an alternative route to reduce fungicide usage

In order to activate induced resistance in the plant, there is a wide range of non-host-specific plant defence inducers, termed 'elicitors', that can stimulate pathogen-inducible defence mechanisms in the plant (Aranega-Bou et al. 2014). Elicitors are able to induce local acquired resistance (LAR), SAR or ISR and may be from different origins, such as biological, chemical or physical (Terry & Joyce 2004; Darras 2011). Some types of biological elicitors, such as PAMPS/MAMPs, can trigger plant defences, such as SAR, by binding to plant membrane-localized pattern recognition receptors (PRRs), which include flagellin, harpin, lipopolysaccharides (LPS), proteins or amino acids, oligogalacturonides, oligosaccharides such as chitin and chitosan, fatty acids, and

peptidoglycans among others (Iriti & Faoro 2009; Chuang et al. 2010; Galletti et al. 2011; Van den Ackerveken et al. 1993). Other type elicitors with proven abilities in crop protection against biotic stress can be from synthetic sources, such as the functional salicylic acid (SA) analogue benzothiadiazole (BTH) and probenazole or its metabolite saccharin (Boyle & Walters 2006; Ahmad et al. 2010). Elicitors can help the plant to triggers its defences in a focussed, specifically-targeted way, which could lead not only to better crop protection, but also substantially reduce the need for conventional pesticide/fungicide use. Moreover, elicitors enable the plant to respond to actual pathogen threats in a broad-spectrum and more efficient manner without damaging other species or the environment and with reduced hazard to the operator. Furthermore, pathogens do not develop resistance as elicitors target plant defences rather than killing pathogen directly, a common issue with other toxic crop protectants such as fungicides. Their effective application requires understanding of their associated defence responses (gene expression) and mode of action in the plant and the agronomy of the crop, which can ultimately benefit crop protection strategies. However application of such crop protectants can be hindered in practice by legislative bureaucracy and knowledge-gaps (Wargent et al. 2013).

The aim of this PhD study is to help the horticulture industry to find new alternatives crop protectants to reduce fungicide usage, by determining the mode of action of specific elicitors, to characterise their molecular function and to investigate their role in priming crops against *Botrytis cinerea*, the fungal pathogen causative agent of the devastating gray mould disease, using *S. lycopersicum* and *B. cinerea*, as a practical model crop and necrotrophic fungal pathogen.

1.6.5 Costs of elicitor-induced resistance

It is clear that induced resistance can be an effective tool to improve plant defence systems and help them to survive microbial challenges. It is well-known also that activation of inducible defences can also carry some fitness costs and trade-offs effects on plant development and reproduction (Ton et al. 2009; Walters & Heil 2007), and this has to be taken into account. To date, relatively few studies have quantified the costs of induced resistance, although there is evidence that in some cases application of chemical synthetic elicitors, such as 2,6- dichloroisoniciotinic acid (INA) and benzothiadiazole (BTH) and the non-protein amino acid elicitor β-Aminobutyric Acid

(BABA), can lead to negative effects on further plant development (Walters & Heil 2007; Zhong et al. 2014; van Hulten et al. 2006). Furthermore, more studies about the application of elicitors, such as the phytohormone jasmonic acid (JA) or its methyl ester relative methyl jasmonate (MeJA) showed costs in terms of reduced seed production and leaf growth or a delayed flowering and fruiting (Darras 2011; Redman et al. 2001). However, it seems that priming is a more effective strategy in terms of enhanced resistance with modest fitness costs to the plant compared with direct induction of defences (Wang et al. 2015). In most cases, studies indicate that plants in the 'primed state' efficiently fight back against pathogen challenge without major effects on seed and plant development (Ton et al. 2009), therefore, priming-based induced resistance represents a beneficial strategy under disease pressure that balances the plant fitness costs and trade-offs (van Hulten et al. 2006; Walters et al. 2008).

In order to achieve priming benefits it seems that there is a dose-dependent effect where low-dose 'priming agent' application can enhance resistance and even protect the plant trans-generationally against biotic and abiotic stresses, balancing plant energy resources with a positive outcome towards defences with minimal costs in plant development (Luna et al. 2012; Król et al. 2015a; Wang et al. 2015). Indeed, studies have already shown that low elicitor doses can enhance resistance to pests without interfering with crop production (Redman et al. 2001).

1.7 *Solanum lycopersicum* as a model plant in crop protection

Tomato (*S. lycopersicum*) belongs to the third most economically important plant taxa, after legumes and grasses with a worldwide production of over 162 million tons in 2012 (FAOSTAT 2014). Tomato belongs to the *Solanaceae* family, which comprise more than 3000 species, some of which are economically very important for the agriculture industry, including, potato (*Solanum tuberosum*), aubergine (*Solanum melongena*), and tobacco (*Nicotiana tabaccum* and *Nicotiana benthamiana*). Tomato is considered to have high nutritional and yield values but fungal pathogens remain an important challenge to yield (Ahmad et al. 2014).

During the last decade, together with *A. thaliana* and potato, tomato has become one of the most important model research plants, and together with *Nicotiana attenuate,* it has been a model crop in wound-related response studies (Scranton et al. 2013). In addition, tomato has served as a model organism to study fruit ripening (Giovannoni

2004) and has emerged as an informative experimental system to characterize the molecular regulation of the ripening-related susceptibility to pathogens, in particular to necrotrophic fungi, such as *B. cinerea* (Powell et al. 2000; Flors et al. 2005). Unlike other model plants with relatively small number of pathogens, such as *A. thaliana*, tomato is a susceptible species to many pathogens of different types, including fungi, nematodes, viruses, bacteria and insects, which makes the tomato pathosystem an excellent model for studying plant-pathogen interactions (Arie et al. 2007).

Tomato is an important model organism also for genetic and molecular studies (Expósito-Rodríguez et al. 2008) and the tomato genome was published in Nature on May 31st, 2012, culminating years of work by the Tomato Genome Consortium, a multi-national team of scientists from 14 countries (Sato et al. 2012; Lin et al. 2014). Due to this joint work, it is currently known that common tomato has 12 chromosomes with a modest diploid genome size of 950 Mb as well as it has short generation time, has abundant genetic resources, such as mutants collections, microarrays, etc., making it an excellent crop model to study induced resistance, pest and pathogen infections of dicots among others disciplines and thereby contribute to minimizing food losses due to pests and pathogens (Ahmad et al. 2014).

1.7.1 Tomato-*Botrytis cinerea* interaction

As mentioned, tomato is considered one of the most important model crops to study plant-pathogen interactions, including tomato-*B. cinerea* (Arie et al. 2007). Due to the well sequenced genome and genetic resources, many studies have helped to decipher pathways and novel genes involved in tomato resistance against this fungal necrotroph (Li et al. 2014; Liu et al. 2014; Li et al. 2015; Li et al. 2015; Liu et al. 2014; Blanco-Ulate et al. 2013; Diaz et al. 2002). Cross-hybridization experiments with wild resistant tomato relatives, such as *Solanum lycopersicoides* and *Solanum habrochaites*, have also contributed to the identification of quantitative trait loci (QTL) and defence and metabolic processes involved in resistance against *B. cinerea* (Finkers et al. 2007; Guimarães et al. 2004; Smith et al. 2014; Chen et al. 2013). Furthermore, -omics and subsequent functional analysis have helped to unveil signalling pathways necessary for tomato resistance against *B. cinerea* (Blanco-Ulate et al. 2013), including transcriptomic profiling of resistant tomato lines, either wild tomato relatives or mutant lines, such as the ABA-deficient tomato mutant *sitiens*.

Sitiens is known as highly resistant to *B. cinerea* (Asselbergh et al. 2007) and has been used for the last decade to decipher tomato defence mechanisms against *B. cinerea*, which appeares to be related to an efficient production of reactive oxygen species (ROS), cell-wall modifications and ethylene production during early stages of the infection (Asselbergh et al. 2007; Sivakumaran et al. 2016; Audenaert et al. 2002).

Furthermore, RNA-seq or microarray technologies have helped to identify specific genes involved in resistance and have given deeper insights of tomato gene defence expression mechanisms (Chen et al. 2013; Smith et al. 2014; Guimarães et al. 2004; Asselbergh et al. 2007). However, more large-scale –omics studies and further combination of pairs of these technologies (e.g. transcriptomics and metabolomics) are needed to determine and ultimately decipher the complex interaction of tomato-*B.cinerea*. This may contribute a better understanding of the role of defence phythormone pathways (Glazebrook 2005; Asselbergh et al. 2007) in this pathosystem, including biosynthesis, transcriptional regulation and signal transduction of ethylene (ET), salicylic acid (SA), jasmonic acid (JA), and abscisic acid (ABA), that appear to be necessary for partial resistance against *B. cinerea* in the early development of the infection (Diaz et al. 2002; Audenaert et al. 2002).

1.7.2 Use of chitin-based elicitors for induced resistance

To date, few studies have investigated elicitors and/or priming agents mode of action in tomato-induced resistance against *B. cinerea* through large-scale transcriptomic analysis (Finiti et al. 2014) and full understanding of tomato defence mechanisms against this pathogen is lacking (Asselbergh & Höfte 2007; Diaz et al. 2002). Chitin, the major component of fungal cell-wall, can be recognized by plant PRRs, serving as a microbe-associated molecular pattern (MAMP) (Miya et al. 2007). During the last decade, the use of chitin-based elicitors to induce immune responses in humans, animals and plants has raised considerably (Cheung et al. 2015; Bueter et al. 2013; Romanazzi, Feliziani, et al. 2013; Muñoz & Moret 2010; Pichyangkura & Chadchawan 2015; Anusuya & Sathiyabama 2014; El Hadrami et al. 2010).

Chitosan, the deacetylated derivative of chitin, has been extensively used in agriculture to induce resistance in ornamental, cereal, horticultural and medicinal crops against a wide variety of biotic and abiotic stresses (Pichyangkura & Chadchawan 2015). Chitin and chitosan have shown to be effective in protecting various crops, such as strawberry,

tomato and grape against *B. cinerea* (Muñoz & Moret 2010; Romanazzi et al. 2013; El Hadrami et al. 2010).

Numerous studies have shown the diverse mechanisms of action of chitosan to induce immune responses in the plant, including activation of pathogenesis-related (PR) proteins, such as glucanases and chitinases (Muñoz & Moret 2010); activation of reactive oxygen species (ROS)-related enzymes, such as peroxidases, superoxide dismutases and catalases (El Hadrami et al. 2010); induction of H_2O_2 and nitric oxide signalling and possibly gene expression control by chromatin interaction (Pichyangkura & Chadchawan 2015). Chitosan-induced resistance in the plant comprises early immune-related gene expression, as part of the PAMP-triggered immunity (PTI), which include the activation of MAP-kinase (MAPK) signalling, accumulation of cytosolic H+ and Ca2+, production of reactive oxygen species (ROS); synthesis of phythohormones, such as abscisic acid (ABA) and jasmonic acid (JA); and callose accumulation (El Hadrami et al. 2010; Iriti & Faoro 2009; Romanazzi et al. 2013).

1.8 Role of –omics in plant-microbe interaction

In the last decade, huge advances in understanding plant immune system, its evolution and the importance of the cell biology in plant-microbe interaction have been accomplished (Jones & Dangl 2006; Pieterse et al. 2012; Zipfel 2014). –Omics, together with genetic analysis, have become powerful and useful tools to bring new understanding of plant endogenous defence mechanisms (Motion et al. 2015).

1.8.1 Transcriptomics

Gene expression or transcriptomic analysis, such as microarrays/DNA chips, have become a common technique due their relatively low costs compared with other newer and more costly technologies (Feussner & Polle 2015). Transcriptomics have helped to decipher gene expression in different plant tissues under different type of biotic and abiotic stresses. In particular, either small-scale or large-scale hybridization-based microarrays in Solanaceous plants have focused on response to herbivore challenge, wounding and jasmonic acid-triggering elicitors, such as MeJA, and coronatine (Scranton et al. 2013) and various transcriptional profiling studies have been made of tomato-infected tissue (Blanco-Ulate et al. 2013b; Guimarães et al. 2004; Finiti et al. 2014) as well as the effects of abiotic stresses such as salinity (Wargent et al. 2013).

DNA chip technology relies on the availability of a well-sequenced genome database of the studied organism, hence new high-throughput DNA sequencing technologies have arisen that do not depend on the genome data readiness and they can detect transcripts of low abundance, such as RNA-seq (Schuster 2008; Ansorge 2009; Biswas et al. 2014) which uses next-generation sequencing (NGS). RNA-seq has become an useful novel tool that directly determines cDNA sequences throughout millions of short cDNA reads and hence recognizing low abundant transcripts (Li et al. 2012; Zhong Wang, Mark Gerstein 2009). However, DNA hybridization-based analysis, such as microarrays, are also high throughput and relatively inexpensive (Zhong Wang, Mark Gerstein 2009), which make them a suitable tool for transcriptomic analysis of well-sequenced and with genomic information available plants, such as tomato. Moreover, tomato has been used a model crop for many different types of transcriptomic assays (Chen et al. 2013; Sato et al. 2012; Zuluaga et al. 2013; Pombo et al. 2014; Wargent et al. 2013; Zhong Wang, Mark Gerstein 2009) which is an advantage for comparison of specific genes and pathways triggered upon biotic/abiotic stress type of transcriptomic analysis. In this PhD study, cDNA based microarray will be performed for both the plant host (tomato) and pathogen (*B. cinerea*), of which genomic sequence information is available.

1.8.2 Metabolomics and proteomics

Not all transcripts are translated into functional gene products or proteins (Fernie & Stitt 2012) and a wider approach is needed in order to identify key elements involved in plant response to stress, such as proteomics and metabolomics, which bring additional information about plant defences as they represent the end product of the immune system pipeline (Feussner & Polle 2015). Proteomics and metabolomics are a useful tool that have helped to understand plant-microbe interactions further by including the apoplast as a key element of the plant cell/tissue immune response (Figure 1.8) and helped to identify novel molecules in non-model plants as an important part of infected tissue (Feussner & Polle 2015). In addition, proteomics have allowed identification of novel virulence factors involved in pathogenicity (Magnin-robert et al. 2009; González-Fernández & Novo 2010; Espino et al. 2010) which could open new possibilities for integrated crop protection (González-Fernández & Novo 2010), such as pathogen and pathotype diagnostics.

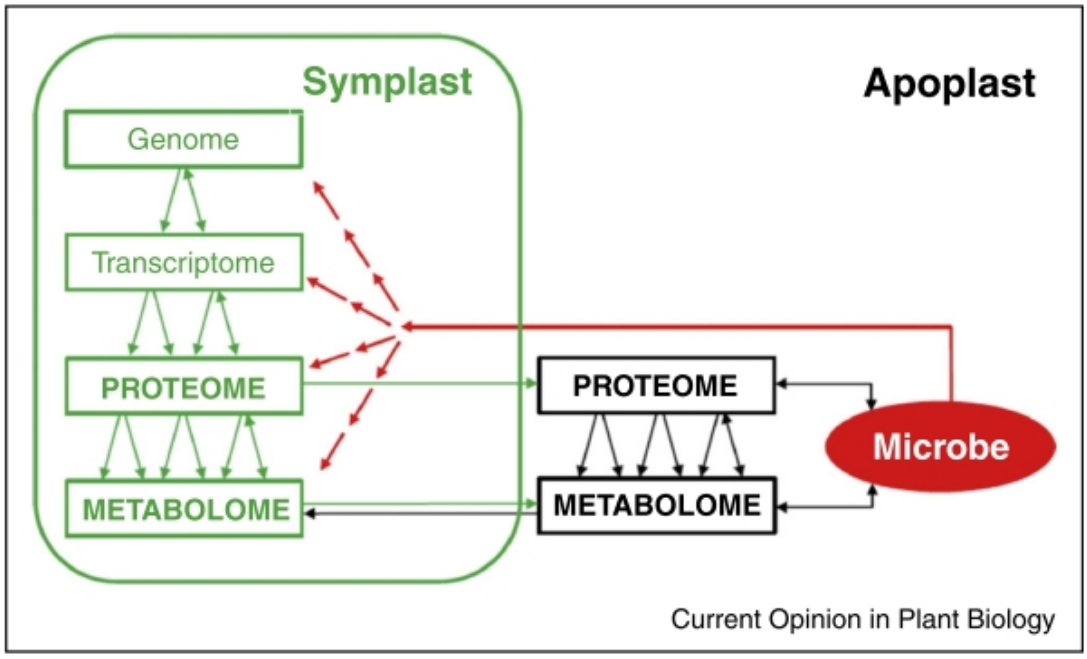


Figure 1.8 Scheme depicting the flow of information and hierarchy of regulatory processes during plant–microbe interactions at different spatial and hierarchical levels. Green: plant-derived levels; red: microbe-derived levels; black: system levels that derive either from the plant or from the microbe or from both (Feussner & Polle 2015).

Currently, genome-based studies can be more robust and add value to the analysis by including proteome or metabolome to the transcriptome analysis. The large diversity of plant-microbe interactions means that there is a great abundance of different biochemical responses (Allwood et al. 2008), including plant produced antimicrobial metabolites to reduce insect herbivory and pathogen infections, such as the accumulation phytoalexins, pathogen-related (PR) proteins and proteinase inhibitors (Kliebenstein et al. 2005; Pluskota et al. 2007; Hückelhoven 2007).

Of all the "-omics" techniques, metabolomics constitute the ultimate level that defines modifications in metabolite fluxes that are not fully controlled by gene expression and/or the proteome (Allwood et al. 2008). Analysis of the metabolome constitutes one of the newest –omics approach to study plant-pathogen interaction (Heuberger et al. 2014) hence metabolomics can be a great tool to study pathogen-related manipulation of phythormone pathways through exploiting the high sensitivity of gas chromatography-mass spectrometry (GC-MS) or liquid chromatography-mass spectrometry (LC-MS) analysis (Forcat et al. 2008). Furthermore, metabolomics allows identification of small molecules and, together with proteomics, they usually represent the end products of the

defence-related signalling cascades and hence they are closer to the plant pathogen-caused phenotype (Feussner & Polle 2015).

1.8.3 Reverse genetics to approach the gene function and the patho-phenotype

In this approach, reverse genetics can be a potent tool to discover the function of a gene by analysing the phenotypic effects of specific engineered gene sequences. Reverse genetics has greatly increased our understanding of gene and protein functions in plant signalling cascades in response to biotic and abiotic stresses (Dodd et al. 2010). In addition, due to reverse genetics the importance of primary metabolism and organelle-dependent signalling has been revealed in defence responses (Bolton 2009; Jung & Chory 2010). Thus, reverse genetics stands as an essential complementary technique to –omics in order to approach the gene/protein/metabolite roles on the patho-phenotype. Indeed, many studies have already demonstrated plant gene roles in the patho-phenotype through the transformation of overexpression or silenced KO lines in model plants like *A.thaliana*, *S.lycopersicum*, *N. benthamiana* and *N. tabaccum* (X. Li et al. 2015; Li, Zhang, et al. 2014; D. Li et al. 2015; Liu, Ouyang, et al. 2014; de las Mercedes Dana et al. 2006; Lionetti et al. 2007; La Camera et al. 2011; Scalschi et al. 2015; Luna et al. 2014) or through the transformation of pathogen strains and performing pathogenicity/infection assays (Scheffer & Tudzynski 2006), which can benefit of using novel gene editing techniques such as CRISPR/Cas.

1.9 Summary of Ph.D project aims

Concluding, this Ph.D project aims to identify and characterise resistance elicitor's mode of action in inducing tomato resistance against fungal necrotroph *B. cinerea*, to determine whether chitosan functions as a priming agent in tomato-*B. cinerea* pathosystem and to investigate the molecular response of tomato to chitosan, the pathogen and to the combination of chitosan and pathogen.

Hence, to achieve this a combined approach will be followed, which includes induced resistance phenotypical/pathogenicity assays and a molecular study that will encompass microarray-based gene expression studies following recognition of common fungal pathogen molecules (PAMPs) by the plant to identify marker genes involved in tomato induced resistance. Quantitative RT-PCR assays will be developed for informative marker genes identified from the microarray study to measure the amplitude and

response time of plants following treatments with the elicitor and the pathogen. A metabolomics approach will investigate the role of phytohormone pathways in tomato defence, elicitor-IR and in response to the pathogen infection which will ultimately add value to the transcriptomic analysis. Finally, reverse genetics will be implemented from selected candidate genes from the transcriptomic analysis in order to investigate their role in plant resistance against *B. cinerea*.

2 Chapter 2. Effects of elicitor-Induced Resistance against *Botrytis cinerea*: Searching for a priming agent

2.1 INTRODUCTION

Plants are able to cope against pathogen attack by the more or less efficient activation of their immune system. In the fight against pathogens, such as *Botrytis cinerea*, plants have developed defence activation mechanisms, including cell-wall-related defences, that can be a potent tool to combat pathogen infection in the early and crucial stages of the infection (Hématy et al. 2009). Plant's initial defence mechanisms, such as callose deposition, the plant polysaccharide, and reactive oxygen species (ROS), can play an important role in reducing and/or delaying pathogen penetration and giving the plant "more time" to display its late acting and fine-tuned defences, such as phytohormone signalling pathways, transcriptional regulation and chromatin/DNA modifications. Callose, the high–molecular weight β-(1,3)-glucan polymer, is considered as an important factor for plant penetration resistance, acting as a physical barrier against invading pathogens such as *B. cinerea* (Hückelhoven 2007). Another important plant initial defence mechanism is the production of reactive oxygen species (ROS), such as H_2O_2, by the plant (Temme & Tudzynski 2009). However, production of ROS can be manipulated by necrotrophic fungi such as *B. cinerea* in order to promote hypersensitive response (HR) and local cell-death to facilitate fungal infection (Govrin & Levine 2000; Smith et al. 2014). In contrast, the abscisic acid-deficient *sitiens* tomato mutant, which is highly resistant to *B. cinerea*, displays a strong induction of hydrogen peroxide during the early stages of the necrotroph infection, suggesting that plant resistance against *B. cinerea* might involve a time-dependent fine-tuning of ROS- and cell-wall-dependent defences (Asselbergh et al. 2007). Both these initial defence mechanisms, callose and ROS production, can be induced by the application of elicitor molecules in the plant (Galletti et al. 2008; Luna et al. 2011; Iriti & Faoro 2009). Activation of plant endogenous defences by elicitors can result in induced resistance (IR), a broad-spectrum resistance against a wide range of biotic and abiotic stress (El Hadrami et al. 2010; Luna et al. 2016; Barilli et al. 2015; Król et al. 2015).

Elicitors are able to mimic pathogen-inducible defence mechanisms in the plant (Aranega-Bou et al. 2014). Elicitor-mediated induced resistance can be asscociated to an effect on hormone-dependent defence gene expression, such as activation of pathogenesis-related (PR) proteins, which are proteins produced by the plant in the event of a pathogen attack. Very well-known example of PR proteins is the pathogenesis-related protein 1 (PR-1), which is one of the plant defence genes that has been extensively used as a marker for salicylic acid (SA)-mediated defence and systemic and local acquired resistance (LAR, SAR) in various model plants (Laird et al. 2004), such as *S. lycopersicum*, *N. tabacum* and *A. thaliana* (Cohen 2002). Other examples of of PR-proteins are proteinase inhibitor (PIs), synthesized in response to either herbivore or pathogen attacks (Farmer & Ryan 1990) that act as anti-nutritive defence compounds (Pluskota et al. 2007). Moreover, PI-I and PI-II, two jasmonic acid (JA)-dependent PIs, have shown to be required for resistance against necrotrophic pathogens (El Oirdi et al. 2011; Blanco-Ulate et al. 2013).

Elicitors can induce resistance in tomato and other crops, and subsequently can enhance the plant basal resistance after perception of elicitor signals against pathogen attack (Luna et al. 2016). One of the main mechanisms of induced resistance is priming (Finiti et al. 2014; Conrath et al. 2002), which implies activation of systemic responses only when the pathogen reaches the infection site (Aranega-Bou et al. 2014). Previous studies have identified the long-lasting effects and adaptive benefits provided by elicitor-induced priming to treated plants that can be transmitted to future generations (Worrall et al. 2012). Some examples include the chemical priming elicitor β-aminobutyric acid (BABA), which can induce resistance even 28 days after treatment (dat), termed long-lasting resistance, in *A. thaliana* against *Hyaloperonospora arabidopsidis* (*Hpa*) and its priming effect can still be detected in the next generation (Luna, López, et al. 2014). The phytohormone jasmonic acid (JA), together with BABA, applied as a seed treatment in tomato, is also able to induce long-lasting priming against herbivores and powdery mildew (*Oidium neolycopersici*) at 8-9 weeks after treatment (Worrall et al. 2012) or against *B. cinerea* (Luna et al. 2016), however both JA and BABA also had an impact on plant growth at high concentrations. It is widely known that there are costs and trade-offs associated with induced resistance (Redman et al. 2001; Luna et al. 2016; Walters & Heil 2007; van Hulten et al. 2006). Nevertheless, few studies have examined this in detail in crop systems. Luna et al., 2016

showed that a soil drench of BABA at high concentrations (10 mM) and JA (1 mM) on 1-week-old tomato seedlings abolished plant growth and had lethal effects. Thus, to achieve a more efficient defence strategy that is less costly in terms of plant fitness, it is important, when using elicitors, to assess the effect of the concentration not only on the activation of plant endogenous defences, but also on the growth and stress tolerance of the plant.

The constant plant-pathogen co-evolution has become a major motivation for the conservation or generation of genetic diversity in both the plant and the pathogen species (Stukenbrock & McDonald 2009). Polymorphisms identified among the many *A. thaliana* accessions sequenced (http://1001genomes.org/) reflect the natural genetic variation even within a plant species, which is in part an effect of the plant adaptation to abiotic and biotic stress (Zhang et al. 2013). Through time, commercial cultivation of crop species, such as tomato, under relatively low biotic pressure environments (e.g. greenhouses and nurseries), may have increased its susceptibility to pathogens due to the loss of resistant (R) genes. However, various studies have reported quantitative resistance in wild tomato relatives to the necrotrophic ascomycete *B. cinerea* (Finkers et al. 2007; ten Have et al. 2006). In this Chapter, two cultivars of tomato were tested in order to investigate genetic variation in long-lasting (17 dat) elicitor-induced resistance against the fungal pathogen *B. cinerea.*

The work reported in this chapter aims to identify and characterise the ability of candidate elicitors to efficiently protect two different tomato cultivars against *B. cinerea*, assess their costs to plant growth and thereby identify new potential priming agents for use in IPM strategies. Moreover, this chapter shows the high effectiveness of one of the candidate elicitors in inducing resistance in various Solanaceous and one brassicaceous crops against *B. cinerea*. Based on the phenotypic disease development (pathogenicity) assays, antifungal activity assay, ROS production assessments and callose deposition analysis, here I describe the identification and further characterisation of a microbe-associated molecular pattern (MAMP) as a novel priming agent among various elicitors, namely ChitoPlant, a water-soluble low MW chitosan formulation.

2.2 MATERIALS AND METHODS

2.2.1 Long-lasting elicitor-induced resistance in tomato against *Botrytis cinerea*: Screening for a resistance phenotype

This study was performed as part of an internship in the lab of Prof. Jurriaan Ton and Dr. Estrella Luna (Sheffield University). Seeds of two tomato cultivars (cvs.), Moneymaker and Motelle, were placed in Petri-dishes containing wetted tissue paper, and maintained at 28°C in the dark for 4 and 3 days, respectively, to stimulate germination. Germinated seeds of each cultivar were planted in plant cell propagators containing Scott's M3 soil (ICL Levington Advance M3 High Nutrient Potting that contains majority peat with a small amount of coir added to aid water uptake) and cultivated under tomato standard growth conditions (16h- 8 h/day-night cycle; 23 °C/ 20 °C) for one week. Each propagator contained 12 seedlings of each cultivar. Seedlings were then soil drenched or foliar sprayed with resistance elicitors (REs), to the following final concentrations.

- Control: foliar sprayed ddH_2O (distilled water) + 0.02% Silwet L-77 (adjuvant/surfactant) + 0.05% ethanol + soil-drench of ddH_2O (150 mL water per tray)
- DL-β-aminobutyric acid (BABA, Sigma) soil drenched (5 mM stock solution = 0.5mM final concentration)
- Benzo (1,2,3)-thiadiazole-7-carbothioic acid S- methyl ester (BTH, BION; Syngenta, Basel, Switzerland) (1 mM) + 0.02% Silwet L-77 (surfactant/adjuvant) + 0.05% ethanol foliar sprayed
- Methyl-jasmonate (MeJA, Sigma) (0.1 mM) + 0.02% Silwet L-77 foliar sprayed
- Combination of BTH+MeJA (0.25 mM+0.1 mM)+ 0.02% Silwet L-77 foliar sprayed
- Chitosan1 (water soluble commercial chitosan formulation termed 'ChitoPlant') 1% w/v + 0.02% Silwet L-77 foliar sprayed: Low molecular weight chitosan (~83.5 kDa) with a 10.6 % acetylation degree (DA), provided by ChiPro (Younes et al. 2014; Romanazzi et al. 2013). ChitoPlant

was kindly provided for experimental purpose by Chipro GmbH, Biotechnology company in Bremen, Germany

- Chitosan2 (a Naturcrop project formulation) (0.1 % w/v) + 0.02% Silwet L-77 foliar sprayed: This chitosan formulation was made by Naturcrop (Adrian Newton, personal communication)

One-week-old plantlets were treated with the different elicitors and one week later roots were washed to remove elicitors, and seedlings were transplanted to ~200 mL individual pots. Eight seedlings were selected and used for each treatment.

Seventeen days after elicitor treatments (long-lasting defence induction), leaves of every plant were excised and prepared for infection in detached leaves assay. Detached leaves were infected with *B. cinerea* as described in the pathogenicity assay (see below) with some major modifications:

2.2.2 Pathogenicity/infection assay

Infection assay was performed according to Worrall et al., 2012 protocol with some modifications. Briefly, active 4-5-week-old *Botrytis cinerea* R16 (Faretra & Pollastro, 1991), kindly provided by Professor Jurriaan Ton (Sheffield University), hyphae growing was cultured on potato dextrose agar (PDA) in 9cm triple vent Petri dishes were kept in the dark at room temperature. Once *B. cinerea* was sporulating, 20 mL of ddH_2O with 0.01% Tween 20 (adjuvant) was added to the Petri dish and it was subsequently scratched with a spatula to release and harvest spores. Spore concentration was then counted with a cell counter haemocytometer and adjusted to 5 x 10^4 spores/ mL (Figure 2.1) (or adjusted to 2 x 10^4 spores/ mL for the rest of the infection assays due to *B. cinerea* R16 strain's high level of aggressiveness). As a final inoculum solution, 3.3 mL of 1 M glucose (freshly prepared/autoclaved) + 2.2 mL of 0.1 M KH_2PO_4 (pH 5.0) (freshly prepared/autoclaved) were added and the incubation time was reduced to 10-15 min in order to decrease the aggressiveness of the fungal strain.

A minimum of 8-12 individual plants (biological replicates) were used per treatment. Whole leaves (fully formed but neither oldest nor newest ones) from approximately 4-week-old plants were excised. Detached tomato leaves were placed into plastic trays with damp tissue and covered with tin foil and black plastic bags to avoid moisture loss. To keep detached leaves 'healthy' and moist for longer periods, petioles were wrapped

in moist tissue as they touched directly the tray's damp tissue, but the leaves were supported on the Petri dishes. Once leaves were set up, they were challenged by inoculation with 6-8 µL (depending on the leaflet size) droplets of *B. cinerea* spores. Detached infected leaves were incubated under high humidity (~90-95%) in the dark at 22°C for 5 days. Once necrotic symptoms appeared 2 days after spore inoculation, infection was scored at 2, 3 and 4 days after inoculation by measuring the diameter (mm) of the lesions with an electronic ruler. Statistical tests (ANOVA and/or pairwise t-test) were performed (Genstat 18th edition) in order to look for significance.

2.2.3 Short-duration chitosan-induced resistance in *Solanum lycopersicum*, *Solanum melongena*, *Nicotiana benthamiana* and *Arabidopsis thaliana* against *Botrytis cinerea*

On the following short-duration elicitor-induced resistance experiments, pathogen infections (pathogenicity assays) were performed at 4-5 days after elicitor treatment (dat).

2.2.3.1 Effects of chitosan-induced resistance in *Solanum lycopersicum* against *Botrytis cinerea*

Tomato cv. Moneymaker seeds were placed into propagators containing Bulrush peat (Bulrush pesticide-free black peat, low nutrient and low fertilizer mix) and a layer of the artificial growth substrate vermiculite on the top and left to incubate at 20 °C for 1-2 weeks. Germinated seeds were transplanted to individual pots containing Bulrush soil (pesticide-free compost mix, nutrient and fertilizer rich and with a water-retaining polymer designed to increase the water retaining property) and grown in either a growth cabinet or a glasshouse under standard conditions (16h-8h/ day-night cycle; 23°C/20°C) before use. Four-week-old cv. Moneymaker plants were treated 4 days prior fungal infection with ddH_2O solution, 0.01%, 0.1% and 1% w/v of chitosan in 0.01% Tween20 by spraying the solution onto the plants. Four days after treatment, 2-3 leaves per plant were excised and subsequently infected with a spore solution of *B. cinerea* (2 x 10^4 spores/ mL) by drop inoculation as described in infection/pathogenicity assay above. Infection was scored at 3 and 4 days after inoculation by measuring the diameter of the lesions with an electronic ruler.

2.2.3.2 Effects of chitosan-induced resistance in *Solanum melongena* against *Botrytis cinerea*

Aubergine (*Solanum melongena*) cv. Black Beauty seeds were placed into propagators containing Bulrush peat (see above) and a layer of vermiculite on the top and incubated at 20 °C for 1-2 weeks until germination. Seedlings were then transplanted to individual pots containing Bulrush soil (see above) and grown in either a growth cabinet or glasshouse under standard conditions (16h-8h/ day-night cycle; 23°C/ 20°C). Four-week-old plants were treated, 4 days prior fungal infection, with ddH_2O solution, 0.01%, 0.1% and 1% w/v of chitosan (in 0.01% Tween20) by spraying the solution onto the plants as above. Four days after treatment, 2-3 leaves per plant were excised and subsequently infected with a spore solution of *B. cinerea* (2×10^4 spores/ mL) by drop inoculation as described above. Infection was scored at 3 and 4 days after inoculation by measuring the diameter of the lesions with an electronic ruler.

2.2.3.3 Effects of chitosan-induced resistance in *Nicotiana benthamiana* against *Botrytis cinerea*

N. benthamiana (wild-type) seeds were placed into propagators containing Bulrush peat and a layer of the artificial growth substrate vermiculite on the top and left to incubate at 20 °C for 1-2 weeks. Germinated seeds were transplanted to individual pots containing Bulrush soil and grown in glasshouse under standard conditions (16h-8h/ day-night cycle; 26° C/22° C) before use.

Four-week-old plants were foliar sprayed 4 days prior fungal infection with (i) ddH_2O solution + Tween20 0.01% (adjuvant/surfactant) and (ii) chitosan (ChitoPlant) 0.01% w/v) + Tween20 0.01% by spraying the solution onto the plants as above. Four days after treatment, 2-3 leaves per plant were excised and subsequently infected with a spore solution of *B. cinerea* (2×10^4 spores/ mL) by drop inoculation as described above. Infection was scored at 3 and 4 days after inoculation by measuring the diameter of the lesions with an electronic ruler.

2.2.3.4 Effects of chitosan-induced resistance in *Arabidopsis thaliana* against *Botrytis cinerea*

This study was conducted with the collaboration of Dr. Estrella Luna (Sheffield University). *A. thaliana* Columbia-0 plants were mass-seeded on soil (Sheffield compost, 2/3 of Scott's M3 soil and 1/3 sand), grown in cabinet and cultivated under

Arabidopsis standard growth conditions (8h-day (21ºC) and 16h-night (18ºC) cycle at ~60% relative humidity (RH). Ten-day-old plants were transplanted to another pot with a total of 5 plants per pot. Five-week-old plants were treated 4 days prior fungal infection with ddH_2O solution, 0.01%, 0.1% and 1% w/v of chitosan (in 0.01% Silwet L-77) by spraying the solution onto the plants to just before run-off. Four days after treatment plants were infected with *B. cinerea* as described above and infection was scored at 3 and 4 days after inoculation by measuring the diameter of the lesions with an electronic ruler.

2.2.4 *Bacillus spp*. -induced systemic resistance (ISR) assay on tomato against *Botrytis cinerea*

Firstly, 3-week-old tomato cv. Moneymaker plants, 20 plants per Bacillus strain, grown in pesticide-free compost, were soaked (only the compost/roots) with 2.5 L of bacterial culture at 1 x 10^7 cells for one hour with a solution of *Bacillus subtilis* wild-type (WT) strain NRS1473 (3610, sacA::Phy-spank-gfp) (Hobley et al. 2013) and the *Bacillus spp*. GB03 biocontrol strain. Tomato plants were removed from the pots at 0, 24 and 72 hours after treatment/inoculation and their roots were gently washed in ddH_2O and PBS to remove the compost. Once dried, roots were weighed and ground to a powder using a mortar and pestle. Root solutions were then plated onto LB + Kanamycin Petri dishes and left overnight for counting colonies and subsequently calculating CFU/g root.

Once *Bacillus spp*. strains colonization ability was evaluated, 20 plants per strain of 3-week-old tomato plants grown in Bulrush soil, were soaked with 2.5 L of bacterial culture at 1 x 10^7 cells for one hour in a solution of the *Bacillus subtilis* wild-type (WT) strain NRS1473 and the *Bacillus* spp. GB03 strain. Four days after inoculation, leaves of tomato plants were excised for infection/pathogenicity assay (see above) using spore inoculum concentration at 2 x 10^4 spores/ mL and lesion diameters were measured to assess their resistance phenotype at 3 and 4 dpi.

2.2.5 Effects of chitosan-induced resistance in *Arabidopsis thaliana* against *Hyaloperonospora arabidopsidis*

A. thaliana Columbia-0 plants were mass-seeded on soil (Sheffield compost, see above), grown in cabinet and cultivated under Arabidopsis standard growth conditions (see above). Ten-day-old plants were transplanted to another pot with a total of 5 plants

per pot. Once *A. thaliana* Col-0 plants were 5-week-old, they were infected with the biotrophic pathogen *H. arabidopsidis* (Hpa) treated with ddH_2O solution or 0.01% w/v of ChitoPlant (in 0.01% Silwet) by spraying the solution onto the plants. Four days after ChitoPlant treatment, plants were infected with Hpa by spraying an inoculum containing 1 x 10^5 spores/ mL. Disease was scored at 5 days by classifying trypan blue-stained leaves in different categories of disease colonization. The categories are (i) Class I, Healthy leaf, no Hpa growth; (ii) Class II, Hpa growth less than 25% of the leaf; (iii) Class III, Hpa growth more than 25% of the leaf with no sporangiophores and (iv) Class IV, Hpa growth with sponrangiophores. Trypan blue stains dead cells and fungus/oomycete mycelium and spores. Leaf samples were submerged in trypan blue stain (50 mL Falcon tube), boiled for 1-2 minutes, cooled then washed once with ddH_2O. Leaves were submerged in chloral hydrate and de-stained overnight. One day later leaf samples were mounted on slides using 60% glycerol for microscopy visualization.

2.2.6 Chitosan antifungal activity on *Botrytis cinerea* spore germination and hyphal growth (*in vitro* assay)

B. cinerea mycelial growth and spore germination (*in vitro* assay) was performed using potato dextrose agar (PDA) as culture media amended with different concentrations of chitosan (1, 0.1, 0.05, 0.01 % w/v). PDA was autoclaved and then ChitoPlant and the fungicide Switch (as positive fungicide control) (1, 0.1, 0.05, 0.01 % w/v) amounts were added directly to the PDA. Solutions were shaken until dissolved and then 15 mL was added per Petri dish (plates). Once media + chitosan/switch solutions were cooled down, a 5 mm diameter agar plug of actively growing *B. cinerea* (BcR16 strain) mycelia was added per plate (5 plates per treatment) to test ChitoPlant fungicide effect on *B. cinerea* mycelia growth. Finally, a 15 µL droplet of *B. cinerea* spores (2 x 10^4 spores/ mL) was added per plate (5 plates per treatment) as well to test for the effect chitosan on spore germination. Plates were covered with parafilm and then incubated under controlled conditions (darkness and 24°C). PDA itself was used as a regular fungal growth condition control. After incubation for 4 days, the mean radial growth of the fungus was determined by measuring the fungal colonies in two perpendicular diameters and calculating the mean diameter.

2.2.7 Initial plant defence mechanisms in elicitor-induced resistance in *Solanum lycopersicum* against *Botrytis cinerea*

2.2.7.1 Basal callose deposition induced by BABA, MeJA, BTH+MeJA and BTH, 'Chitosan1' (ChitoPlant) and 'Chitosan2' in tomato cvs. Moneymaker and Motelle.

Tomato cvs. Moneymaker and Motelle were grown under standard conditions in trays containing 12 seedlings of each cultivar. Seedlings 1-2-weeks old were treated with:

1. Control: ddH_2O + 0.02% Silwet (surfactant) + 0.05% ethanol (control 1), foliar sprayed + soil-drench of ddH_2O (150 mL water per tray)
2. BTH (1 mM) + 0.02% Silwet + 0.05% ethanol, foliar sprayed
3. 'Chitosan1' (ChitoPlant) 1% w/v + 0.02% Silwet + 0.05% ethanol, foliar sprayed
4. 'Chitosan2' (0.1 % w/v) + 0.02% Silwet + 0.05% ethano, foliar sprayed l
5. MeJA (0.1 mM) (which was dissolved in ddH_2O + 0.05 % ethanol) + 0.02% Silwet, foliar sprayed
6. BTH + MeJA (0.25 mM+0.1 mM) + 0.02% Silwet, foliar sprayed
7. Soil drenched with BABA (5 mM stock solution = 0.5 mM final concentration)

Cotyledons of every treatment were excised one week after treatment and stored in 100% ethanol before aniline blue staining as described previously (Luna et al. 2011). Briefly, cotyledons were incubated for at least 24 h in 95 to 100% ethanol until all tissues were transparent, were washed in 0.07 M phosphate buffer (pH =9), and were incubated for 1 to 2 h in 0.07 M phosphate buffer containing 0.01% aniline blue (Sigma), prior to microscopic analysis. Observations were performed with an epifluorescence microscope with UV filter (BP 340 to 380 nm, LP 425 nm). Callose was quantified from digital photographs by the number of white pixels (callose intensity) or the number of depositions relative to the total number of pixels covering plant material, using ImageJ software.

2.2.7.2 Dose-dependent basal callose deposition induced by ChitoPlant in tomato cv. Moneymaker and *A. thaliana* Col-0

4-week-old tomato plants were treated with ddH_2O-mock solution, 0.001%, 0.01% and 0.1% w/v of ChitoPlant (in 0.01% Tween 20) by spraying the solution onto the plants. Plant material was collected at 3 and 6 hours (hat), then 3 and 5 days after treatment (dat), fixed in 96% ethanol before aniline blue staining as described above and callose deposition was analysed with an epifluorescence microscope with UV filter (BP 340 to 380 nm, LP 425 nm) and quantified as above.

A. thaliana Col-0, plants were mass-seeded on soil (Sheffield compost, see above), grown in a growth cabinet under Arabidopsis standard growth conditions described above. Ten days-old plants were transplanted to another pot with a total of 5 plants per pot. Six-week-old plants were treated with ddH_2O-mock solution, 0.01%, 0.1% and 1% of ChitoPlant (in 0.01% Silwet) by spraying the solution onto the plants. At one and two days after treatment, plant material was collected in 96% ethanol and callose deposition was analysed as above (aniline blue staining).

2.2.7.3 Pathogen-induced callose deposition in elicitor-treated tomato plants

Tomato cvs. Moneymaker and Motelle 1-2-week-old seedlings (12 per cultivar) were treated with:

1. Control: ddH_2O + 0.02% Silwet + 0.05% ethanol (control1), foliar sprayed + soil-drench of ddH_2O (150 mL per tray) (control 2)
2. Chitosan1 (ChitoPlant) 1% w/v + 0.02% Silwet + 0.05% ethanol, foliar sprayed
3. Chitosan2 (0.1 % w/v) + 0.02% Silwet + 0.05% ethanol, foliar sprayed
4. MeJA (0.1 mM) + 0.02% Silwet, foliar sprayed and soil drenched with BABA (5 mM stock solution = 0.5 mM final concentration)

Seventeen days after elicitor treatments (long-lasting defence induction) leaves of every plant were excised and prepared for infection/detached leaves assay. Leaves were settled into trays and challenged by droplet inoculation with *B. cinerea* spores. Finally, double staining (aniline blue + calcofluor) was performed at 2 dpi in order to see pathogen-induced callose deposition in all treatments. Briefly, leaf discs surrounding infected tissue were harvested and fixed in 96% ethanol and left overnight prior to staining. Leaves were placed on 0.05% aniline blue solution and 0.001% calcofluor for

approximately 15 minutes, staining solution was replaced with fresh aniline blue (without calcofluor) and incubated at room temperature overnight in the dark. Slides were prepared in fresh aniline blue solution and view under 365 nm excitation light with DM 400 LP. Measurements were carried out at 2 dpi at a spore concentration of 2 x 10^4 spores/ mL.

2.2.7.4 ChitoPlant and MeJA-induced peroxidase (POD) activity

Four-week-old tomato cv. Moneymaker seedlings (3 plants per cultivar) were foliar sprayed with:

1. Control: ddH_2O + 0.01% Tween 20 (surfactant)
2. Chitosan (ChitoPlant) 0.01% w/v + 0.01% Tween 20
3. MeJA (0.1 mM) + 0.01% Tween 20
4. Combination of chitosan+MeJA (0.01% w/v + 0.1 mM) + 0.01% Tween 20

In addition, *sitiens*, the highly resistant to *B. cinerea* tomato mutant (Asselbergh et al. 2007a), which is impaired in the oxidation of ABA-aldehyde to ABA (Audenaert, Meyer, & Hofte, 2002), is known to highly induce POD activity, was used as a positive control. Extracellular peroxidase activity was examined with the tetramethylbenzoidine (TMB) assay described by (Barceló 1998). Briefly, 4 days after treatment, tomato leaves were excised for infection assay and detached leaves were inoculated with two 6-8 µL droplets of *B. cinerea* (BcR16 strain) spore inoculum (2 x 10^4 spores/ mL) (infected) or with the water/control (mock) solution. Finally, tomato leaf discs were harvested surrounding the infection site and fixed in ethanol at 6, 24 hpi (asymptomatic *B. cinerea* infection stage) and 48 hpi.

After subsequent washing in distilled water, the discs were incubated in 1.5 mL of 50 mM Tris-acetate buffer (pH 5.0) containing 0.1 mg/ mL TMB and 0.03% H_2O_2 for 20 min. Peroxidation of the TMB molecule resulted in blue discoloration of both leaf tissue and incubation solution. Peroxidase activity of the discs was determined by measuring the absorbance of the incubation solution at 654 nm by mass spectrophotometry.

2.2.7.5 ChitoPlant and MeJA-induced H_2O_2 production

Four-week-old tomato cv. Moneymaker seedlings (3 plants per treatment) were foliar sprayed with:

1. Control: ddH_2O + 0.01% Tween 20 (surfactant)
2. Chitosan (ChitoPlant) 1% w/v + 0.01% Tween 20
3. MeJA (0.1 mM) + 0.01% Tween 20

Four days after elicitor treatments, tomato leaves were detached and infected with two 6 µL droplets of *B. cinerea* spore inoculum (2 x 10^4 spores/ mL) as described previously.

To evaluate temporal evolution of H_2O_2 accumulation, infected leaves of the different treatments were sampled and stained at 24 and 48 hpi by using 3,3'-diaminobenzidine (DAB) staining. In this protocol, brown precipitates are formed at the sites of H_2O_2 accumulation (Thordal-Christensen et al., 1997). Chlorophyll was removed from the leaf tissue samples with 96% ethanol to evaluate temporal evolution of H_2O_2 accumulation. Leaves were also infiltrated with H_2O_2 solution and ddH_2O as positive and negative controls respectively.

2.2.8 Hormone-related defence gene expression in *Solanum lycopersicum*

Four-week-old tomato cv. Moneymaker seedlings were treated with ddH_2O + 0.01% Tween 20 and MeJA (0.01 mM) (Wang et al. 2015) + 0.01% Tween 20. Five days after elicitor treatment, tomato leaves (3 seedlings per treatment) were excised and they were infected with *B. cinerea* spore inoculum (2 x 10^4 spores/ mL) and mock inoculated (ddH_2O) as a non-infected control. Sample collection was done by harvesting leaf discs with a cork borer surrounding infection area at 8, 24 and 48 hour post infection (hpi). Alternatively, four-week-old tomato cv. Moneymaker plants were foliar sprayed with ddH_2O (control) + 0.01% Tween 20 (surfactant); Softguard (chitin + chitosan, Travena) 1:600 + 0.01% Tween 20, MeJA (0.1 mM) + 0.01% Tween 20 ; BTH (1 mM) + 0.01% Tween 20 and BABA (foliar sprayed) (250 µg/ mL; (Cohen 2002) + 0.01% Tween 20; leaves of 3 plants per treatment (3 biological replicates) were harvested at 3, 9 and 24 hours after treatment (hat) for total RNA extraction.

Samples were then stored in 2 mL tubes at -80 ºC until RNA extraction with RNeasy Plant MiniKit (Qiagen), DNased with TurboDNase (ThermoFisher) and complementary DNA (cDNA) was synthesized from 5 µg of total RNA using Superscript III reverse transcriptase (Invitrogen) and random hexamer/oligo dt primers.

Quantitative real time PCR (qRT-PCR) reactions were performed using SYBR Green Mastermix (QIAGEN) with specific tomato primers (Sigma-Aldrich) (Table A.1 Oligonucleotide primer list), Lox-D, LapA and EF1-α (reference control gene) (Fowler et al. 2009), NPR1 (designed using PRIMER3 software), Pin1 and Actin (reference control gene) (El Oirdi et al. 2011) and PR-1 (Song et al. 2011). Amplification and detection of specific products were performed with the following cycle profile: denaturation step at 95° C for 15 min, 95° C for 15 sec, annealing and extension at 60° C for 1 min. Each qPCR reaction contained three non-template controls. All reactions were run in technical triplicate for each biological replicate and the average values were used for quantification. The relative quantification of target genes was determined using the ΔΔCt method. Gene expression data was relative to the ddH_2O-treated (control) treatment and data represent means of three biological replicates for the qRT-PCR ± standard error of the mean (SEM). Real-time amplification reactions were performed using SYBR Green detection chemistry. Quantitative RT-PCRs were performed using a Opticon Monitor 3 software (Bio-Rad).

2.2.9 Costs of elicitor-induced resistance in *Solanum lycopersicum* seedling development

2.2.9.1 Elicitor-induced resistance effect on tomato cvs. Moneymaker and Motelle growth (relative growth rate (RGR))

Seeds of 2 tomato cultivars (Moneymaker and Motelle) were placed in Petri dishes containing ddH_2O-soaked tissue paper, and maintained at 28°C in the dark for 4 and 3 days, respectively, to stimulate germination. Germinated seeds of each cultivar were planted in plant cell propagators containing Scott's M3 soil (see above) and cultivated under tomato standard growth conditions (16h- 8h/ day- night cycle; 23°C/ 20°C) for one week.

Each propagator contained 12 seedlings of each cultivar. Seedlings were then soil drenched/sprayed with elicitors, to the following final concentrations.

1. Control (ddH_2O) + 0.02% Silwet, foliar sprayed
2. Chitosan1 (ChitoPlant, ChiPro) 1% w/v + 0.02% Silwet, foliar sprayed
3. Chitosan2 (Naturcrop formulation) (0.1 % w/v) + 0.02% Silwet, foliar sprayed
4. MeJA (Positive control) foliar sprayed (0.1 mM) + 0.02% Silwet, foliar sprayed
5. β-amino-butyric acid (BABA) soil drenched (5 mM stock solution= 0.5 mM final concentration)
6. Benzo (1,2,3)-thiadiazole-7-carbothioic acid S- methyl ester (BTH) (1mM) + 0.02% Silwet + 0.05% ethanol, foliar sprayed
7. Combination of BTH + MeJA (0.25 mM+ 0.1 mM) + 0.02% Silwet; foliar sprayed

Plant height was measured with a ruler every two days during seven days after elicitor treatment to determine elicitor-induced growth reduction. After measuring seedlings height, the relative growth rate (RGR) was calculated. RGR was based on the distance between the soil surface and the apical meristem (Luna et al. 2016), using the formula $RGR= (\ln ht_2-\ln ht_1)/t_2-t_1$, where h_2 and h_1 represent plant height (cm) at time points t_2 and t_1 (days), respectively. Bar graphs represent RGR means ± standard error of the mean (SEM; n = 8 - 12).

2.2.10 Resistance elicitor selected concentrations

All the elicitor concentrations were chosen from published articles where they used them at the specified or similar concentrations (see above) to induce resistance in different plant-pathogen systems. BABA and jasmonic acid (JA) were used to induce resistance in tomato cv. Moneymaker against *B. cinerea* at 1mM and 0.1 mM respectively (Luna et al. 2016). BABA, BTH and JA were used by (Ton & Mauch-Mani 2004) in *A. thaliana* against *A. brassicicola* and *P. cucumerina* and (Barilli et al. 2015) used 10 mM and 50 mM for BTH and BABA, respectively, previously proven to be effective in this Pea-*Uromyces pisi* system; (Terry & Joyce 2004) stated that MeJA and BTH been used against *Fusarium semitectum* and *B. cinerea* as post-harvest and field treatments. (Meir et al. 2005; Walters et al. 2005a) studied MeJA against *B. cinerea* and

BTH on wheat and barley against powdery mildew. Finally, chitosan was shown to induce resistance in tomato against *Fusarium oxysporum* f.sp. *radicis-lycopersici* and to induce defence-related gene expression (Pichyangkura & Chadchawan 2015). Furthermore, (Romanazzi et al. 2013; Romanazzi et al. 2013) studied different chitosan formulations at 1 % w/v and BTH in strawberry and grapevine against *B. cinerea*.

2.3 RESULTS

2.3.1 Elicitor-induced resistance in solanaceous crops and Brassicaceae plant *Arabidopsis thaliana*

2.3.1.1 Long-lasting elicitor-induced resistance in tomato against *Botrytis cinerea*. Screening for a resistance phenotype

Tomato cvs. Moneymaker and Motelle are two susceptible genotypes to the aggressive necrotrophic fungus *B. cinerea*. This study aimed to determine whether BABA, BTH, MeJA, the combination of MeJA + BTH and 2 formulations of chitosan are able to induced long-lasting resistance (17 days after elicitor treatment) against *B. cinerea*, thus tomato cvs. Moneymaker and Motelle, were treated with the different resistance elicitors and subsequently challenged with *B. cinerea* as described in the Materials and Methods.

Among all elicitors, only BTH, the salicylic acid (SA) functional analogue, and methyl-jasmonate (MeJA) were able to significantly reduce *B. cinerea* necrotic lesion size at 3 days post-inoculation (dpi) when the pathogen spore inoculum was highly concentrated (5 x 10^4 spores/ mL) (Figure 2.1). However, BABA provided no protection against *B. cinerea*. There were no significant differences in lesion size between the two tomato cultivars (P=0.086) at 3 days post inoculation (dpi), while these differences were significant at 4 dpi (P=0.003) (Figure 2.1b).

To look for a stronger long-lasting elicitor-induced resistance phenotype, due to the high aggressiveness of *B. cinerea* BcR16 strain (Faretra & Pollastro 1991), the concentration of the fungal inoculum was reduced and the protection level of the elicitors was evaluated. The level of resistance induced by all elicitors at both time points resulted in a statistically significant reduction of lesion size, compared to the water-treated control plants, in both cultivars when *B. cinerea* spore inoculum was adjusted at 2 x 10^4 spores/ mL (Figure 2.2). In cv. Moneymaker, all elicitor treatments significantly reduced *B. cinerea* symptom development in a similar manner, in comparison with the water-treated (control) plants at both time points (Figure 2.2a). In

contrast, there were more differences among treatments in cv. Motelle at both time points (Figure 2.2b). In cv. Motelle, BABA and ChitoPlant were more effective against *B. cinerea* than the other treatments, at 3 dpi. In addition, at 4 dpi in cv. Motelle MeJA and both chitosans were slightly more effective than BABA against *B. cinerea*. After 4 days post-inoculation (dpi), all elicitors still significantly reduced *B. cinerea* lesion expansion, in comparison with the control in both cultivars and some differences among treatments were still seen in cv. Motelle (Figure 2.2b).

BABA and the two chitosan formulations significantly reduced necrotic lesion size at 3 dpi in comparison with water-treated control plants, for *B. cinerea* at 2 x 10^4 spores/ mL. At 4 dpi, MeJA and again both chitosan formulations behaved similarly in significantly reducing symptom development compared to control treatment (Figure 2.2b). There were also significant differences among treatments and the two tomato cultivars at 3 dpi (Figure 2.3), although these significant differences were no longer expressed after 4 dpi.

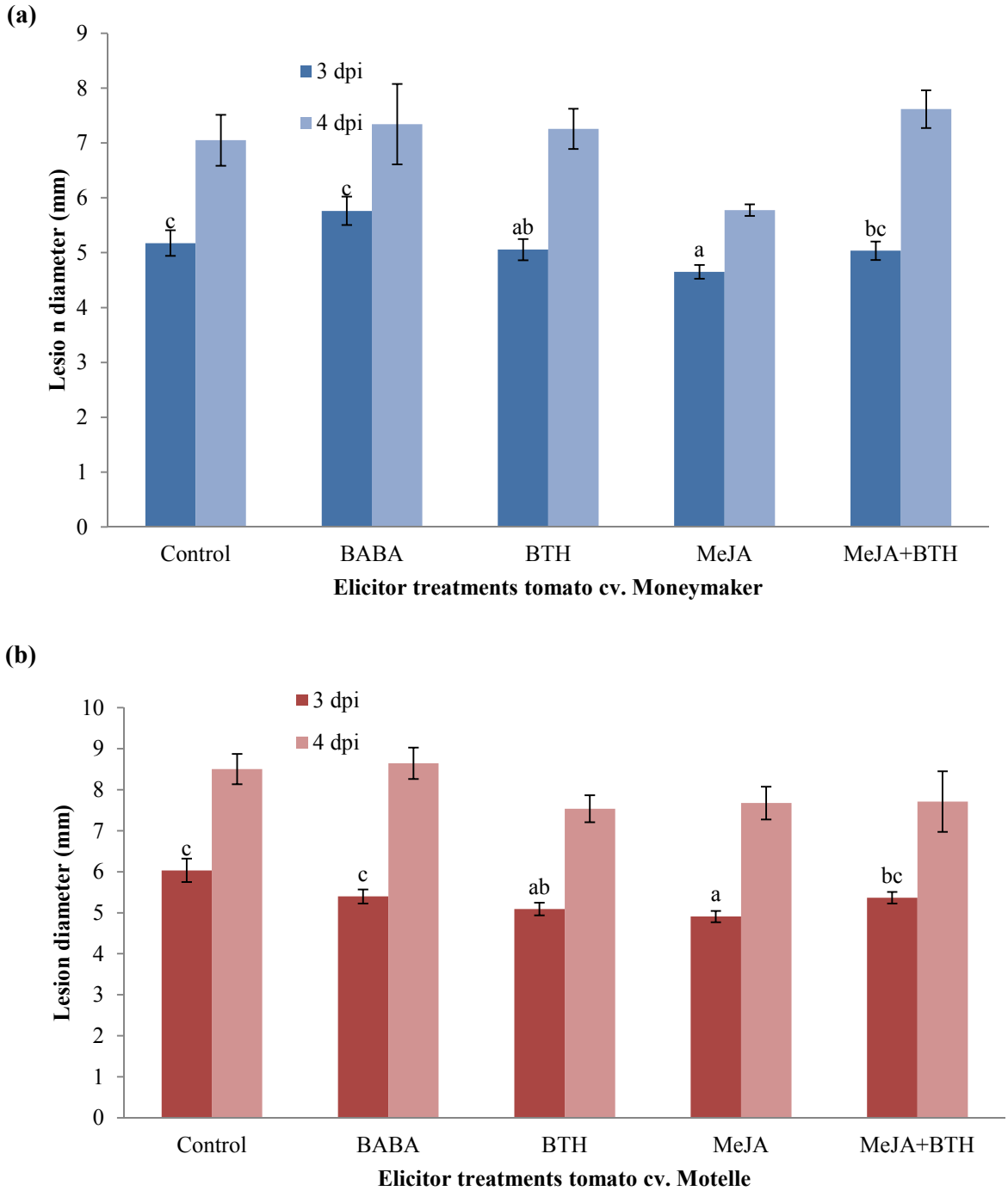


Figure 2.1 Quantification of BABA, BTH, MeJA, MeJA+BTH-induced resistance and ddH_2O-treated (control) against *B. cinerea* (**5 x 10^4 spores/ mL**) in tomato at 3 and 4 dpi. Values presented are means ± SEM. Different letters indicate statistically significant differences among all treatments (ANOVA followed by Fisher's protected least significant difference (LSD) test $P<0.001$ for Treatment, $P=0.086$ for cultivar at 3 dpi; and $P=0.145$ for Treatment; $P=0.003$ for cultivar; $P=0.347$ for Treatment x cultivar at 4 dpi, $\alpha=0.05$); **(a)** Tomato cv. Moneymaker (blue bars); **(b)** Tomato cv. Motelle (red bars)

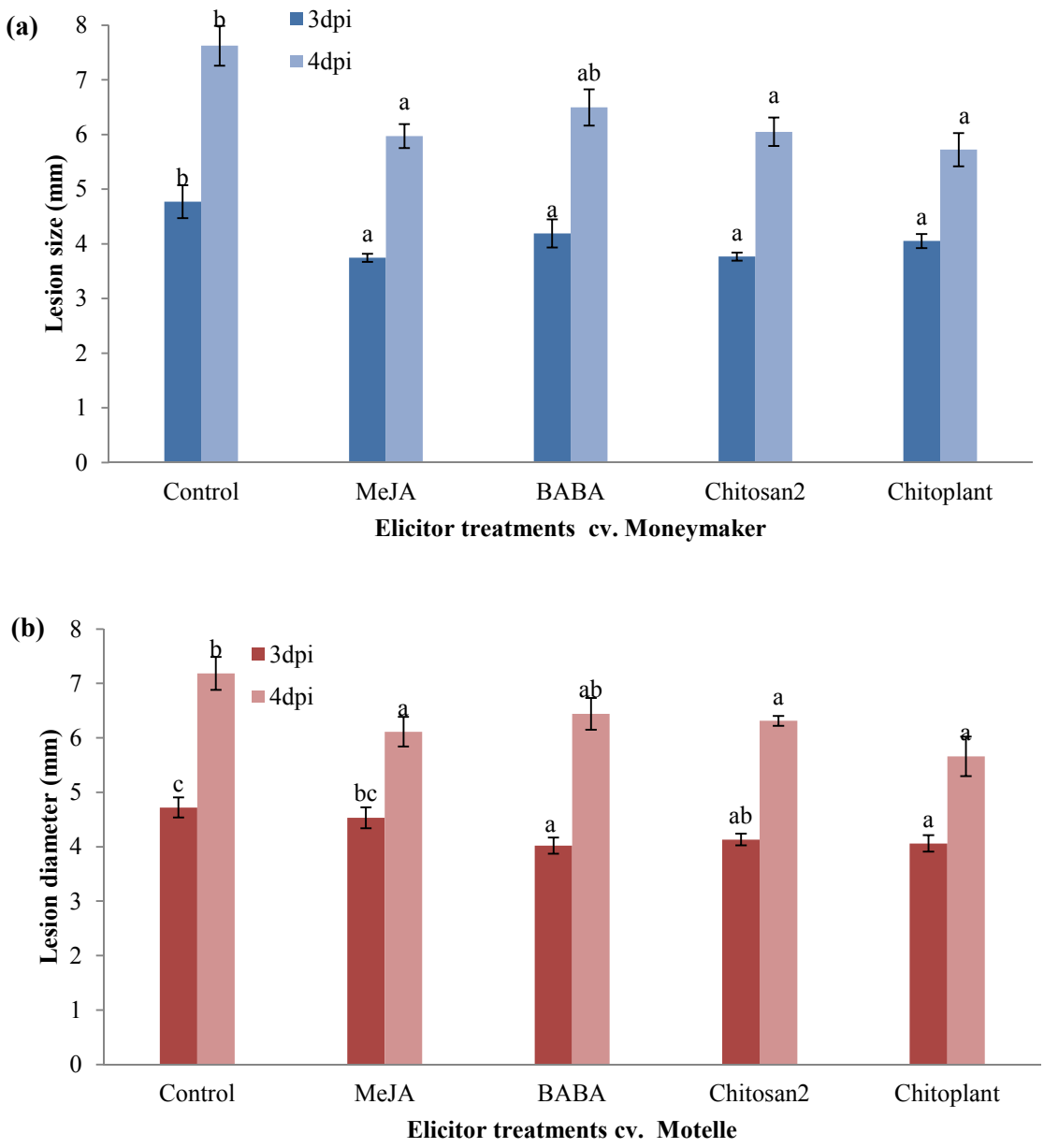


Figure 2.2 Quantification of MeJA, BABA, Chitosan2, commercial chitosan formulation ChitoPlant-induced resistance and ddH_2O-treated (control) against *Botrytis cinerea* (**2 x 10^4 spores/ mL**) at 3 and 4 dpi in tomato. Values presented are means ± SEM. Different letters indicate statistically significant differences among all treatments (ANOVA followed by Fisher's protected least significant difference (LSD) test P=0.004 at 3 dpi and P<0.001 at 4 dpi, α=0.05 for tomato cv. Moneymaker; and P=0.005 at 3 dpi and P=0.009 at 4dpi, α=0.05 for tomato cv. Motelle); **(a)** Tomato cv. Moneymaker (blue bars); **(b)** Tomato cv. Motelle (red bars)

Statistical analysis was also conducted to test whether the candidate elicitors-induced resistance is cultivar dependent. Significant differences were found for cultivar response to elicitor-IR. As seen before, normally tomato cv. Moneymaker expressed a stronger induced resistance (IR) phenotype against *B. cinerea* than cv. Motelle in all treatments,

which was therefore a more susceptible cultivar to *B. cinerea*, except with BABA, where the level of induced resistance was stronger in cv. Motelle than cv. Moneymaker (Figure 2.3). Tomato cv. Moneymaker response to MeJA and Chitosan2 treatments significantly differed from cv. Motelle, expressing a stronger IR phenotype in cv. Moneymaker (Figure 2.3). In contrast, Chitosan1 (ChitoPlant) was able to induce resistance in both cultivars deploying a similar level of resistance/protection against *B. cinerea* independently of the cultivar (Figure 2.3), which makes it a valuable elicitor candidate for further experimentation.

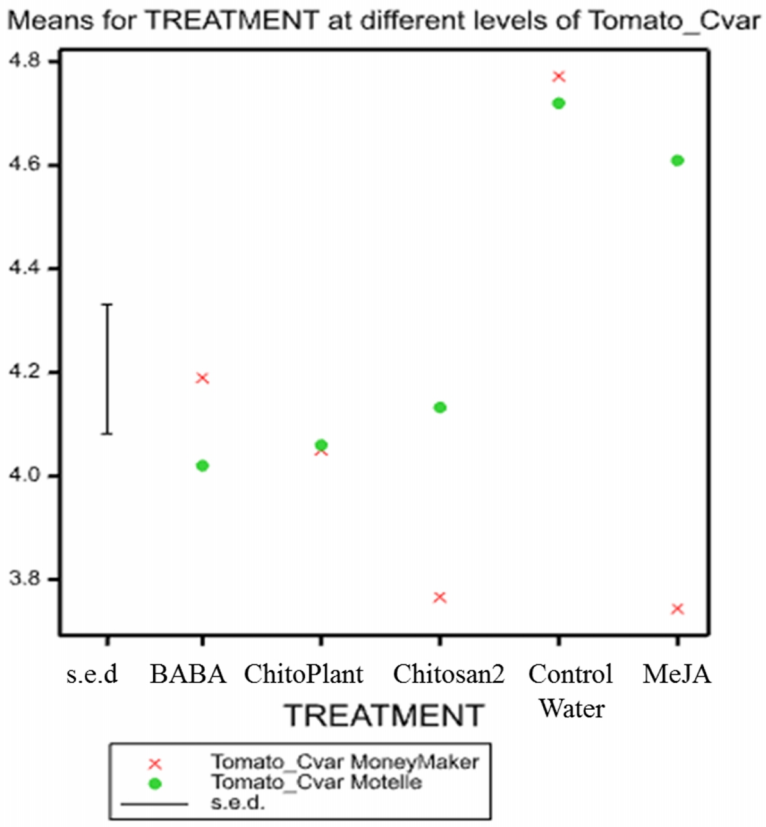


Figure 2.3 Quantification of BABA, MeJA and Chitosan2 and ChitoPlant (ChiPro)-induced resistance phenotype and ddH_2O–treated (control) against *B. cinerea* in tomato cvs. Moneymaker (red spots) and Motelle (green spots) at 3 dpi. Values represented are means (of the necrotic lesion diameter) ± SEM obtained from an ANOVA mean plot ($P<0.05$ for cultivar interaction; treatment x cultivar).

2.3.1.2 Short-duration chitosan and Bacillus spp.-induced resistance in solanaceous crops and *Arabidopsis thaliana* against *Botrytis cinerea*

Among all elicitors previously used, chitosan (water-soluble ChitoPlant formulation) has shown a strong long-lasting (17 dat) induced resistance effect in tomato cvs. Moneymaker and Motelle. Furthermore, chitosan showed a similar protective effect on both cultivars (Figure 2.3) whereas BABA, Chitosan2 and MeJA were cultivar dependent. However, due to the large number of plants and elicitors, chitosan was only used at high concentrations (1% w/v).

Thus, to investigate further chitosan mode of action in other plants and to determine whether chitosan induces short-duration (4-5 dat) resistance in a concentration-dependent manner in tomato, *A. thaliana*, and a range of related Solanaceae species, such as aubergine and *N. benthamiana* against *B. cinerea*, tomato cv. Moneymaker, *A. thaliana* Col-0, *N. benthamiana* (WT) and aubergine cv. Black Beauty plants were foliar treated (see M&Ms), 4 days before fungal infection (short-duration induced resistance), with three different concentrations of chitosan (low, medium and high) and subsequently infected with a spore solution of *B. cinerea* by drop inoculation (pathogenicity assay, M&Ms).

In tomato, pathogenicity assay (see M&Ms) indicated that chitosan significantly decreased necrotic lesion size against *B. cinerea* in all concentrations compared with water-treated control plants at 3 dpi (Figure 2.4). The resistance phenotype provided by chitosan was similar in all concentrations at 2 dpi. However, there were significant differences among chitosan treatments at 3 dpi, where the resistance phenotype induced by chitosan had a dose-depent effect at the two high concentrations (1% and 0.1%) but lowest concentration (0.01%) resistance phenotype was in between 1 and 0.1 % treatments (Figure 2.4).

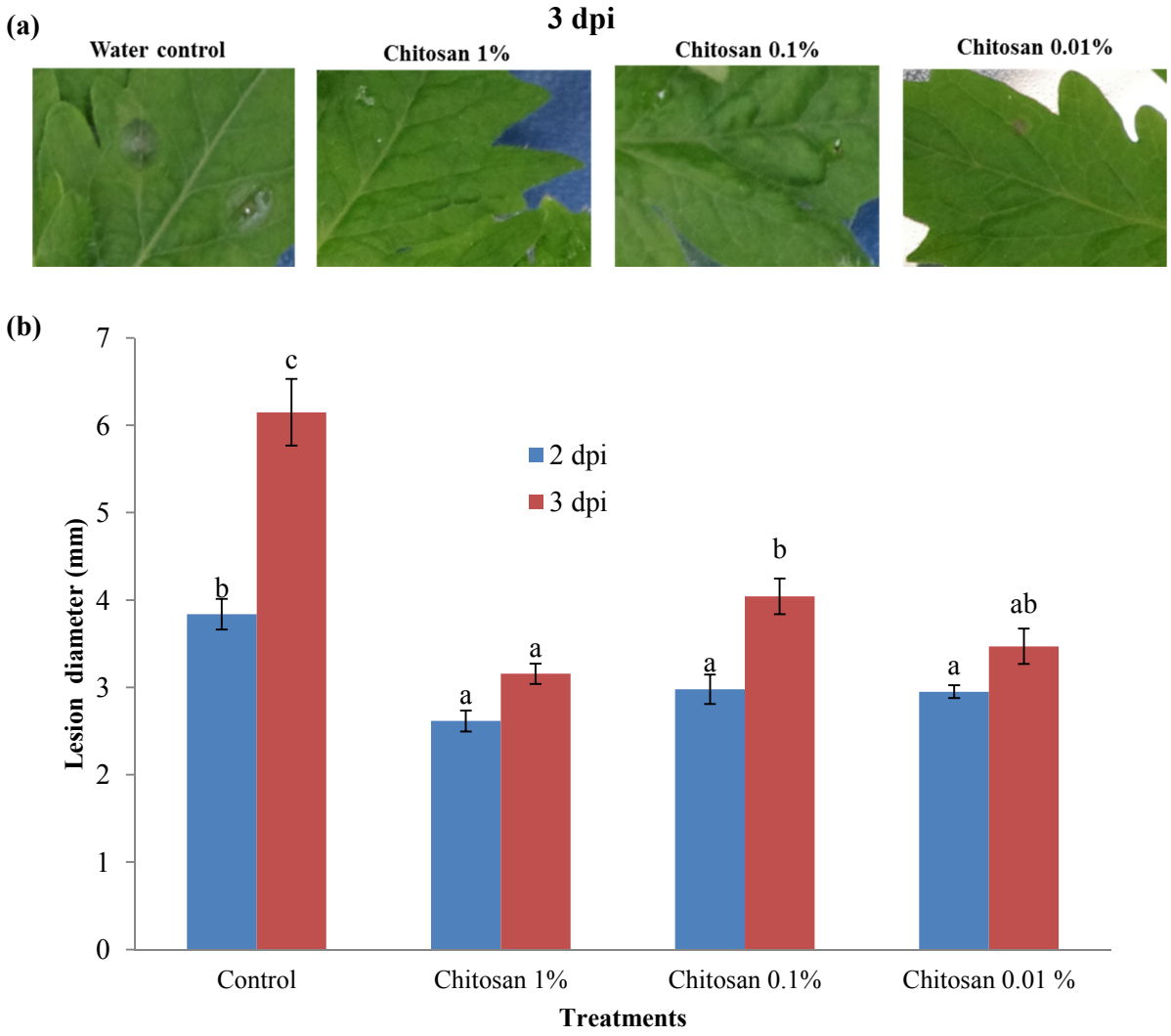


Figure 2.4 Quantification of chitosan-induced resistance in tomato cv. Moneymaker against *B. cinerea*. **(a)** One representative leaflet of eight-twelve replicates is shown for each treatment at 72 hpi. **(b)** Tomato seedlings were treated with chitosan at 3 different concentrations (0.01%, 0.1 % and 1% w/v) and dH_2O-treated (control) at 2 and 3 days post-inoculation (dpi). Values presented are means ± SEM. Different letters indicate statistically significant differences among all treatments (Values presented are means ± SEM obtained from an ANOVA and then pairwise Fisher's protected least significant difference (LSD) test $P<0.001$, $\alpha=0.05$).

In *A. thaliana*, chitosan induced resistance in a concentration-dependent manner. Chitosan significantly decreased *B. cinerea* necrotic lesion size in all concentrations compared to water-treated and infected (control) plants (Figure 2.5). The resistance phenotype decreased with the concentration, chitosan 1% w/v having the strongest

resistance phenotype of the three concentrations and chitosan 0.01% the lowest level of protection but still significant (Figure 2.5).

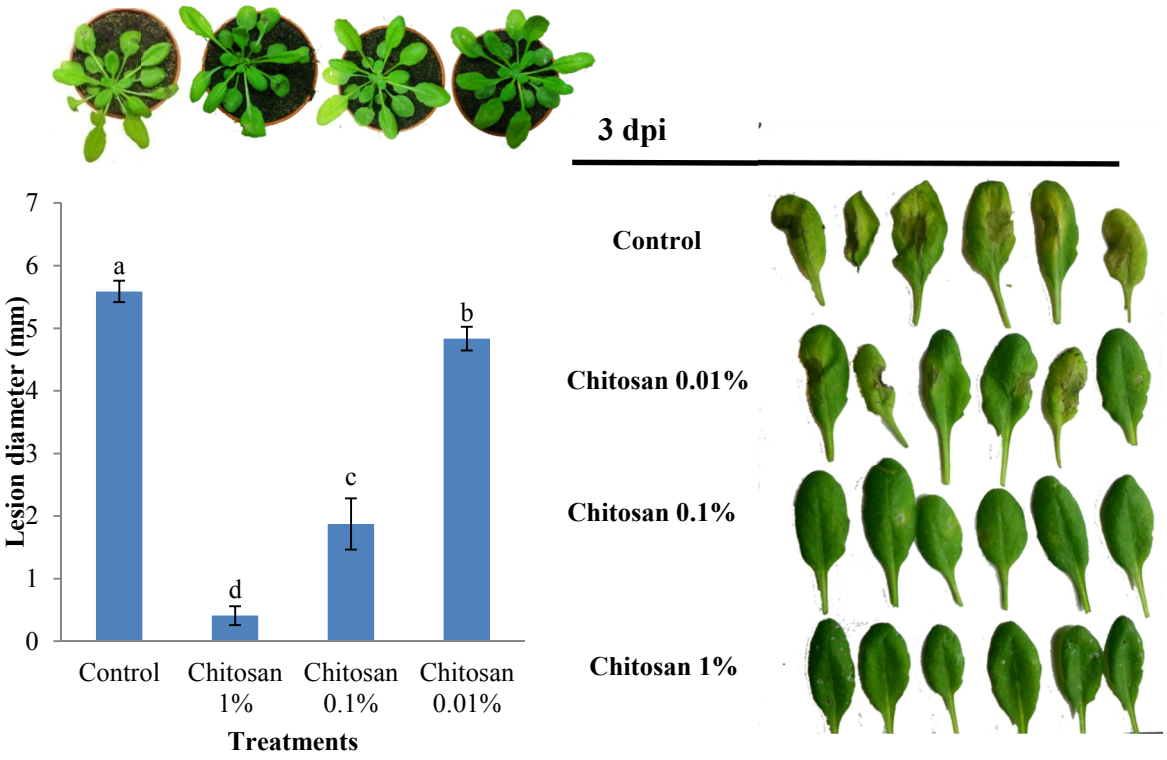


Figure 2.5 Quantification of chitosan-induced resistance at 3 different concentrations (0.01%, 0.1 % and 1% w/v) and ddH_2O-treated (control) in *A. thaliana* against *B. cinerea* at 2 dpi. Values presented are means ± SEM. Different letters indicate statistically significant differences among treatments (Games-Howell's Post-Hoc test $P<0.05$ at 2 dpi). Six representative leaflets of eight-twelve replicates are shown for each treatment at 3 dpi.

In aubergine, chitosan significantly induced resistance in tomato against *B. cinerea* in all concentrations compared to water-treated control plants at 3 dpi (Figure 2.6). Chitosan did not have a concentration-dependent effect as seen in *A. thaliana*. Chitosan 1%-treated plants had significantly bigger lesion (darker coloured lesions with visible fungal growth) size (due to the induced cell-death/cytotoxicity) at 4 dpi than any of the treatments including water-control (Figure 2.6); lesion phenotype was similar as control plants at 5 dpi, with large variability due to chitosan cytotoxic effect observed in some leaves.

However, the two lowest concentrations were able to induce resistance in a concentration-dependent manner at 3 and 5 dpi, where chitosan 0.1% and 0.01% w/v significantly reduced *B. cinerea* necrotic lesion size in aubergine at all time points against *B. cinerea* infection.

These results suggest a possible threshold in chitosan-priming for resistance that depends on its concentration and if exceeded, it may overstress plant defences in benefit of necrotrophic pathogens, which might benefit from dead cells, such as *B. cinerea*.

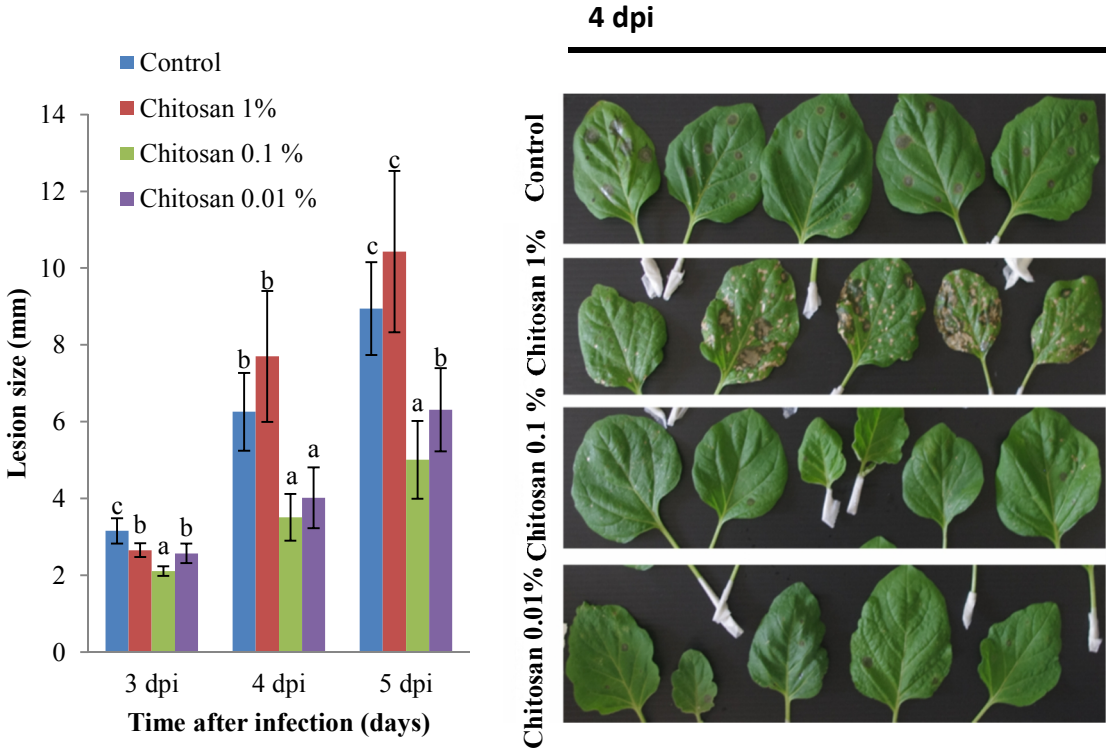


Figure 2.6 Quantification of chitosan-induced resistance at 3 different concentrations (0.01%, 0.1 % and 1% w/v) and ddH_2O-treated (control) in aubergine against *B. cinerea* at 3, 4 and 5 days post-inoculation. Values presented are means ± SEM. Different letters indicate statistically significant differences among treatments (ANOVA followed by Bonferroni's Post-Hoc test $P<0.001$ at 3, 4 and 5 dpi). Five representative leaflets of eight-twelve replicates are shown for each treatment at 4 dpi.

In *N. benthamiana*, due to a more specific aim in this plant, which was to determine whether low-concentrated chitosan solution can induce resistance in this plant against *B. cinerea* and therefore to characterise this further by gene expression and functional analysis studies (Chapter 4). Indeed, as it will be explained later in the Thesis, this concentration was the most suitable to study further chitosan priming properties throughout transcriptomic assay, which makes the results valuable to see whether low-concentrated chitosan induces a resistance phenotype in *N. bentamiana* against *B.*

cinerea. Thus, to determine the effects of chitosan (ChitoPlant) treatment against *B. cinerea*, *N. benthamiana* plants were treated 4 days prior fungal infection, with low-concentrated chitosan and subsequently infected with *B. cinerea* spores for pathogenicity test (M&Ms).

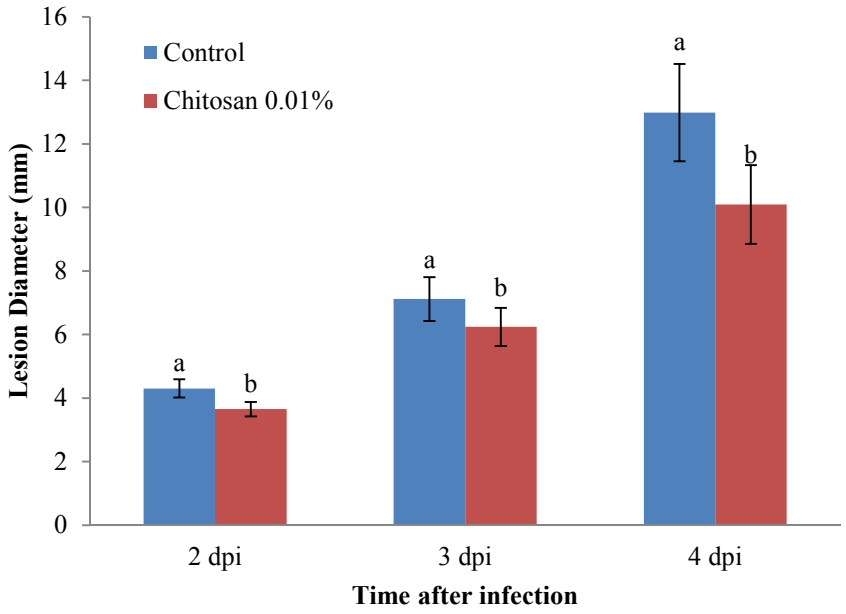


Figure 2.7 Quantification of chitosan-induced resistance against *B. cinerea* in *N. benthamiana* at 2, 3 and 4 days post-inoculation. Values presented are means ± SEM. Different letters indicate statistically significant differences between treatments (Student's t-tests for lesion diameters of leaf infections per time point indicate, $P<0.001$ at 2 dpi, $P=0.007$ at 3 dpi; $P<0.001$ at 4 dpi).

Results indicate that chitosan significantly decreased *B. cinerea* lesion size at 2, 3 and 4 dpi in *N. benthamiana* plants (Figure 2.7).

Finally, after testing chitosan-IR in various plants against *B. cinerea,* I decided to investigate a different approach and type of induced resistance, such as induced systemic resistance (ISR), through studying the role of plant growth-promoting rhizobacteria (PGPR) in tomato defences against *B. cinerea*. *Bacillus subtilis* is a gram-positive plant-associated rhizobacteria that is able to form microcolonies in the plant rhizosphere. First, to evaluate the capacity of two *Bacillus spp.* strains to colonise tomato rhizosphere, tomato cv. Moneymaker plants were soaked with a solution of *Bacillus subtilis* wild-type (WT) strain NRS1473 and the *Bacillus spp*. GB03 strain (kindly provided by Dr. Nicola Holden) and subsequently removed at 0, 24 and 72

hours post-treatment (hpa) for colony counting as described in the Materials and Methods.

Colonisation analysis showed NRS1473 wild-type strain rapidly colonised tomato rhizosphere and maintained the highest values till 24 hours post-inoculation and started to decrease colony forming units (CFU) levels at 72 hours, whereas biocontrol GB03 strain was slower in colonisation at 0 and 24 hours but it retained higher CFU levels after 72 hours (Figure 2.8).

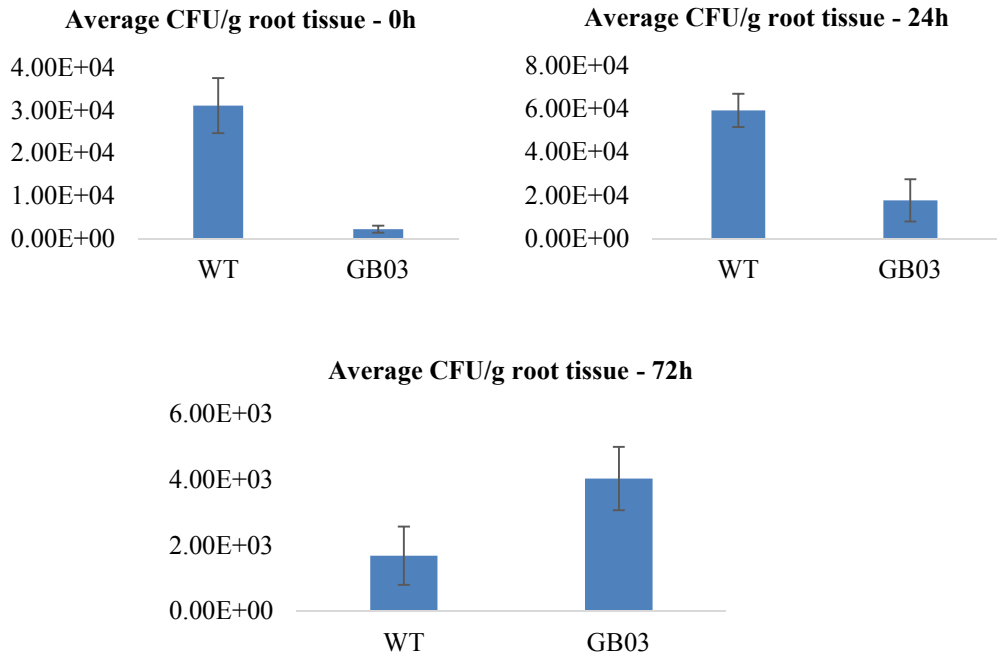


Figure 2.8 Quantification of the average CFU/g root tissue for the *Bacillus subtilis* NRS1473 wild type (WT) and Bacillus spp. GB03 strain soaked plants at 0, 24 and 72 h. Error bars are standard error of the mean. Colonies were counted at 0 h, 24 h and 72 hours after inoculations and CFU/g root was calculated. Values presented are means ± SEM.

Secondly, to investigate the biocontrol properties of two *Bacillus spp.* strains and their ability to induce systemic resistance (ISR) in tomato against *B. cinerea*, tomato plants were soaked (through the roots) with a solution of *B. subtilis* wild-type strain NRS1473 and the *Bacillus* spp. strain GB03 for one hour. Four days after inoculation, leaves of tomato plants were excised for infection assay and lesion diameters were measured to look for resistance phenotype at 3 and 4 dpi as described in the Materials and Methods.

B. subtilis NRS1473 and the *Bacillus spp*. GB03 strain were able significantly delay *B. cinerea* lesion expansion at both time points. However, GB03 strain induced a stronger resistance phenotype at 4 dpi (Figure 2.9) which correlates with the slower and more durable rhizosphere colonisation seen by this strain (Figure 2.8).

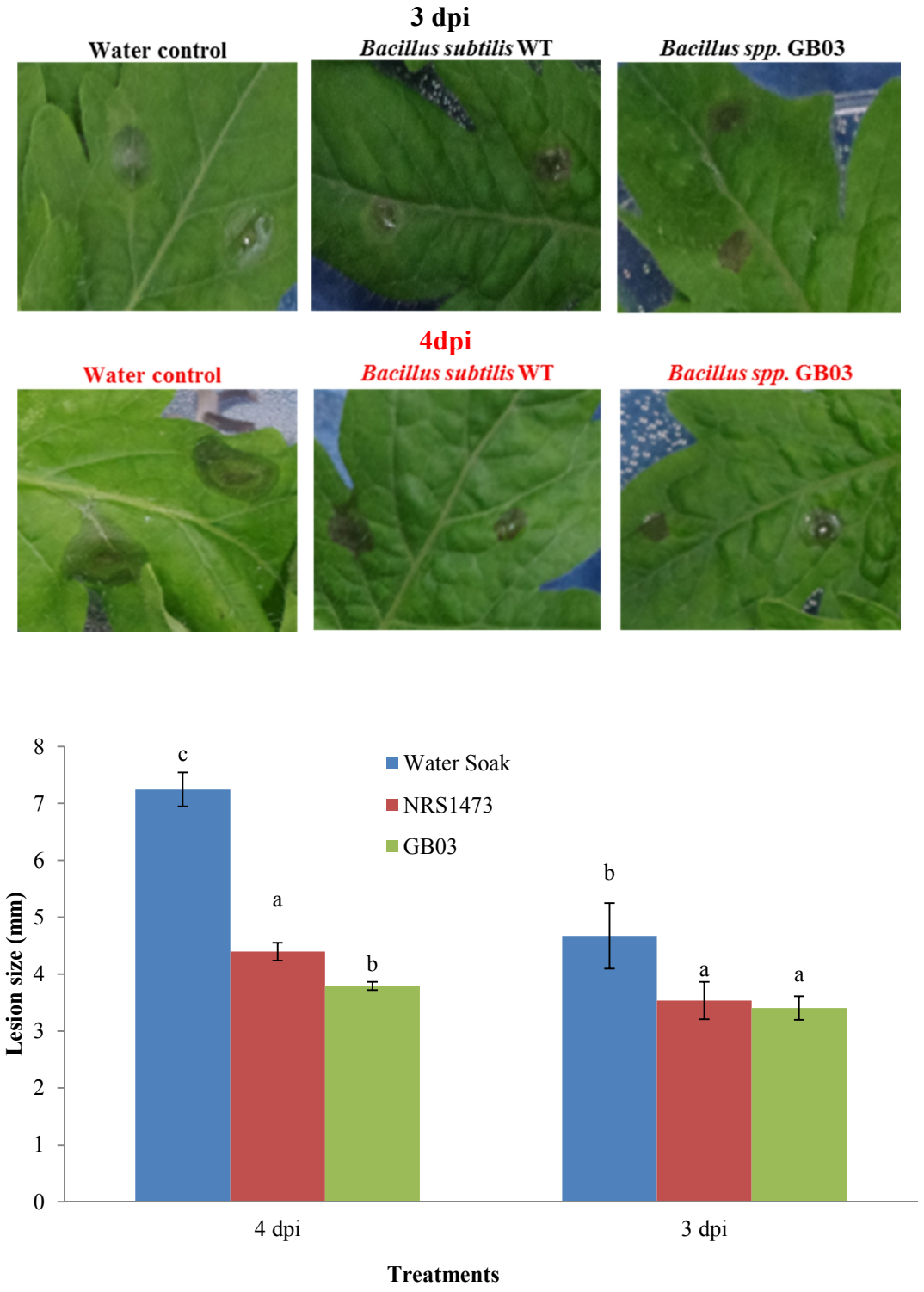


Figure 2.9 Quantification of *Bacillus subtilis* WT strain NRS1473 and *Bacillus spp.* GBO3 –induced systemic resistance (ISR) and ddH_2O soak (control) in tomato cv. Moneymaker against *B. cinerea* at 3 and 4 dpi. Values presented are means ± SEM. Different letters indicate statistically significant differences among treatments (values presented are means ± SEM obtained from an ANOVA and then pairwise Fisher's protected least significant difference (LSD) test $P<0.001$, $\alpha=0.05$).

2.3.1.3 Short-duration chitosan-induced resistance in *Arabidopsis thaliana* against *Hyaloperonospora arabidopsidis*

It has been demonstrated the ability of chitosan in inducing resistance of *S. lycopersicum, S. melongena*, *N. benthamiana* and *A. thaliana* against fungal necrotroph *B. cinerea*. However, it is important to investigate whether this water soluble chitosan formulation (ChitoPlant) can protect plants against other type of pathogens. Thus, with the collaboration Dr. Estrella Luna, this study aimed to determine whether chitosan induces resistance in *A. thaliana* against unrelated plant pathogens. Thus, *A. thaliana* Columbia 0 plants were infected with the biotrophic pathogen *H. arabidopsidi* (*Hpa*) previously treated with ddH_2O solution or 0.01% w/v of chitosan. Four days after chitosan treatment, plants were infected with *Hpa* and disease was scored at 5 days after infection (see M&Ms).

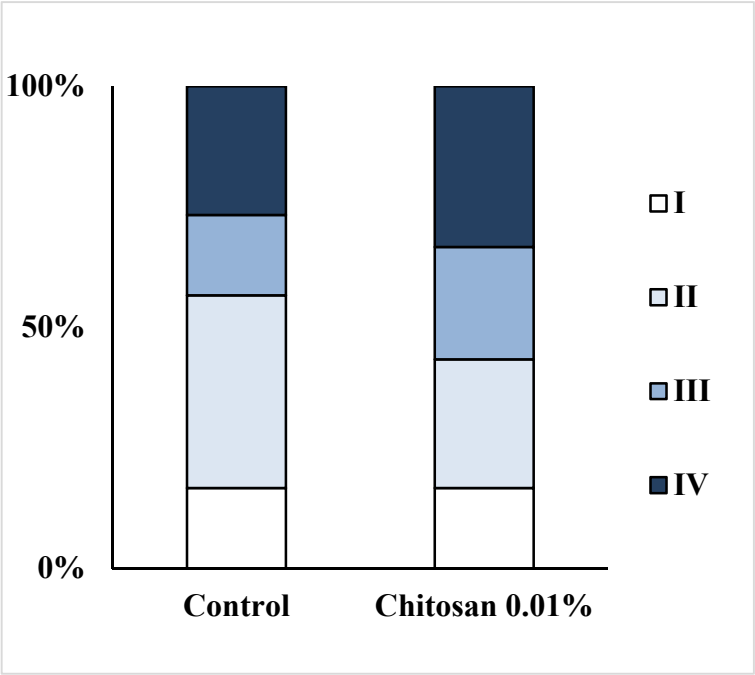


Figure 2.10 Quantification of chitosan-induced resistance at 0.01% w/v and ddH_2O-treated (control) in *A. thaliana* against *H. arabidopsidis* at 5 days post-inoculation. Values presented belong to 4 disease classes. Class I, Healthy leaf, no Hpa growth, Class II, Hpa growth less than 25% of the leaf, Class III, Hpa growth more than 25% of the leaf with no sporangiophores and Class IV, Hpa growth with sponrangiophores. Percentage indicate statistically significant differences (Chi-square p value $P<0.716$).

Both treatments had a similar Hpa infection profile (Figure 2.10) and there were no significant differences between the treatments ($P<0.716$). Thus, we can conclude that chitosan did not induce resistance in *A. thaliana* against the oomycete *H. arabidopsidis.*

This result indicates that chitosan-induced resistance works differently depending on the plant and pathogen species.

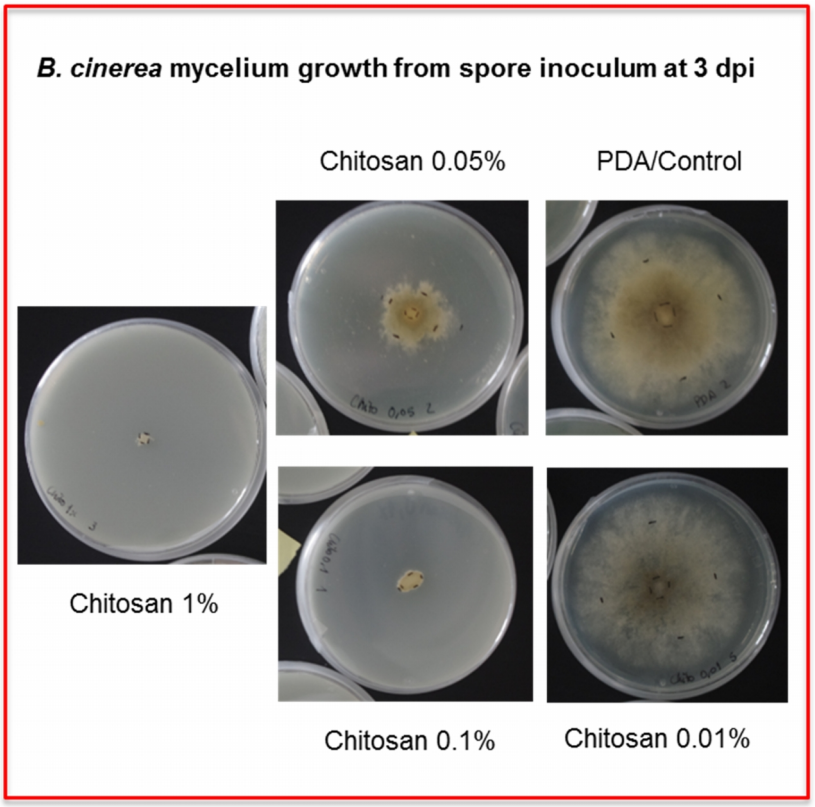
2.3.2 Chitosan antifungal activity on *Botrytis cinerea* spore germination and hypha growth

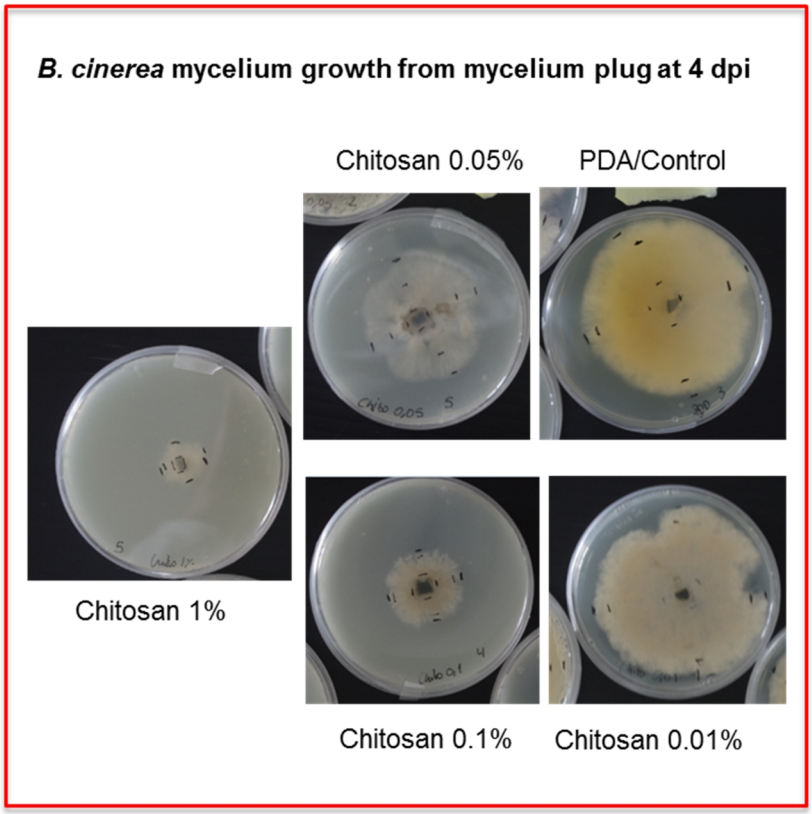
It is well-known that chitosan has antifungal properties (Badawy & Rabea 2014; Romanazzi et al. 2013); however this activity may depend on the physicochemical properties, such as chemical structure, molecular weight (MW) and/or acetylation degree (DA); as well as the concentration. The main focus of this work is to investigate resistance elicitors (e.g. chitosan) role in priming plant own defences rather than having a direct effect on the pathogen such as conventional fungicide mode of action. Thus, in order to investigate the potential direct antifungal effect of chitosan against *B. cinerea*, the effect of chitosan (ChitoPlant) on fungal mycelial growth from actively growing mycelia plugs and from spore inoculum was assessed *in vitro.*

Chitosan had an antifungal effect on hypha growth from both the mycelial plug and spore-inoculated petri dishes in a concentration-dependent manner (Figure 2.11). Both, the highest concentrations (1 and 0.1% w/v) and, to a lesser extent, the medium concentrated (0.05% w/v) had a direct effect on mycelia growth suppression as well as a putative suppression or reduction of spore germination (Figure 2.11). However, the lowest concentration of chitosan (0.01% w/v) did not have any direct antifungal effect, over the time period tested; on *B. cinerea* mycelia and spores inoculum in comparison with control non-treated PDA (Figure 2.11), which suggests a concentration threshold for chitosan-direct antifungal activity against *B. cinerea*. As expected, the positive control fungicide (Switch, Syngenta) supressed both *B. cinerea* mycelia and spores at all concentrations (Figure 2.11).

These results clearly support the hypothesis of the concentration-dependence of priming of elicitors (chitosan in particular), suggesting a concentration threshold in which chitosan might not directly affect fungal growth instead of priming the plant own defence mechanisms against pathogen challenge.

(a)


B. cinerea mycelium growth from spore inoculum at 3 dpi
Chitosan 0.05%
PDA/Control
Chitosan 1%
Chitosan 0.1%
Chitosan 0.01%


B. cinerea mycelium growth from mycelium plug at 4 dpi
Chitosan 0.05%
PDA/Control
Chitosan 1%
Chitosan 0.1%
Chitosan 0.01%

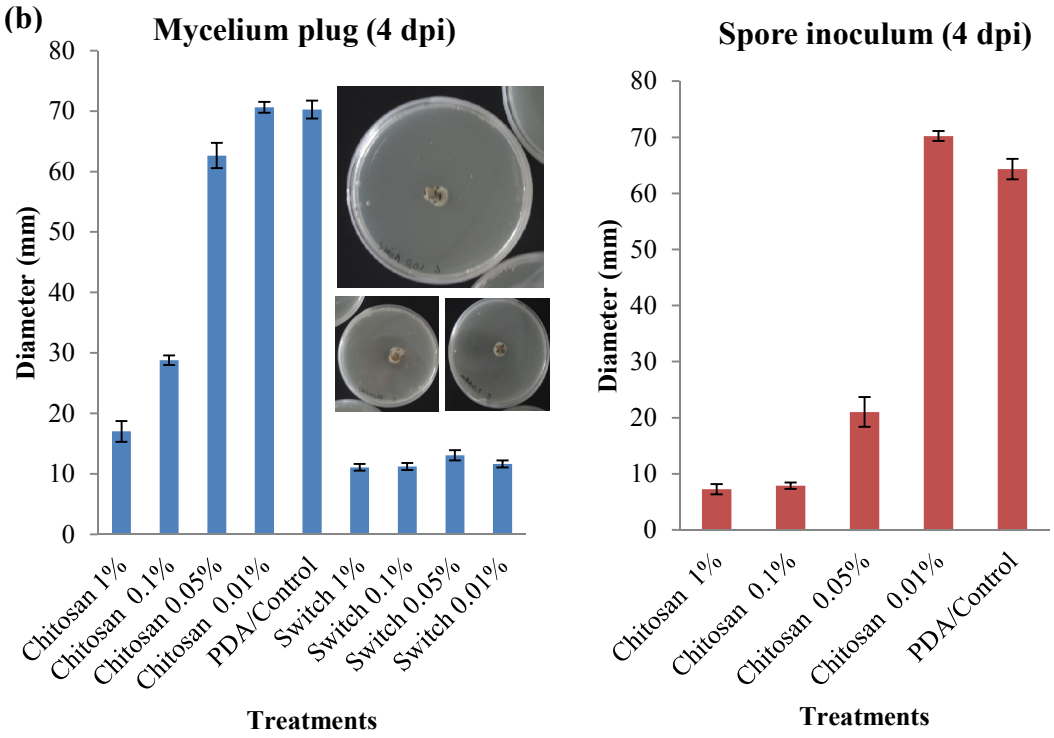


Figure 2.11 Quantification of chitosan-antifungal direct effect on *Botrytis cinerea* in PDA media plates (Petri dishes). 5 mm agar plug of actively growing *B. cinerea* (BcR16 strain) mycelia was added per plate (5 plates per treatment) to test chitosan (Chitoplant, ChiPro) antifungal effect on *B. cinerea* mycelia growth and a 15µL droplet (2 x 10^4 spores/ mL) with *B. cinerea* spores was added per plate as well to test effect chitosan effect on spore germination. Petri dishes were covered with parafilm and were placed in 24°C incubator in the dark. **(a)** Pictures were taken at 3 dpi for the spore-inoculated plates and 4 dpi for the mycelium plug-inoculated plates and **(b)** After incubation for 4 days (4 dpi) the mean radial growth of the fungus was determined by measuring the fungal colonies in two perpendicular diameters and calculating the mean diameter.

2.3.3 Initial plant defence mechanisms in elicitor-induced resistance in *Solanum lycopersicum* against *Botrytis cinerea*

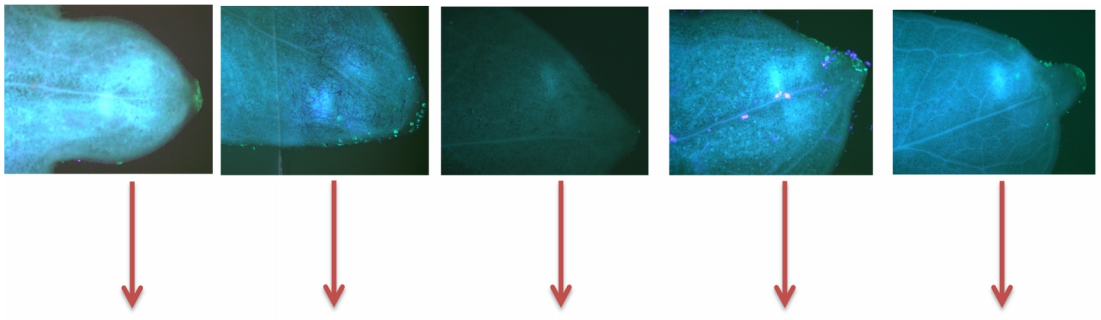
To elucidate whether ChitoPlant induces callose deposition in a concentration-dependent manner, this study aims to show whether different concentrations of the commercial formulation ChitoPlant can induce callose deposition in the tomato cultivar Moneymaker and *A. thaliana* Col-0 in order that ChitoPlant can be used for fundamental studies.

2.3.3.1 Basal callose deposition induction by BABA, MeJA, BTH+MeJA, BTH, Chitosan1 (ChitoPlant) and Chitosan2 in two tomato cultivars

As stated previously, callose is a plant polysaccharide that can reinforce the plant cell-wall and therefore delay pathogen cuticle penetration. This study aimed to assess whether β-aminobutyric acid (BABA), methyl-jasmonate (MeJA), benzo (1,2,3)-thiadiazole-7-carbothioic acid S- methyl ester (BTH, BION), 2 different formulations of chitosan and the combination of BTH+MeJA can induce callose deposition in tomato cvs. Motelle and Moneymaker.

Cvs. Moneymaker and Motelle seedlings were treated with ddH_2O (as a control), MeJA, BTH, MeJA+BTH, Chitosan2, ChitoPlant (Chitosan1) and BABA. Cotyledons for each treatment were excised one week after treatment and stored in 100% ethanol prior to aniline blue staining to dye callose deposition as described in Material and Methods.

Overall, there was the same trend in both tomato cultivars apart from in treatments containing MeJA, where MeJA induced callose and this induction was higher in cv. Moneymaker (MM) than in cv. Motelle (Figures 2.12 and 2.13). The combination of BTH and MeJA also induced callose deposition in tomato cotyledons in cv. Moneymaker (Figure 2.12) and cv. Motelle, although to a lesser extent (Figure 2.13).



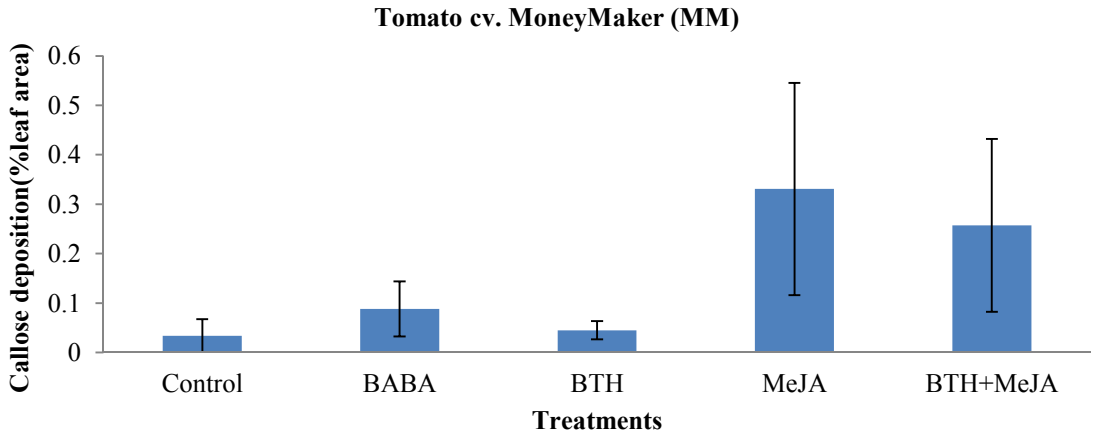


Figure 2.12 Basal callose deposition in tomato cv. Moneymaker leaves after water-control, BABA, BTH, MeJA and MeJA+BTH treatments. Seven days after treatment leaves were harvested for aniline blue staining. Pictures were taken under fluorescence microscopy at 4x magnifications (bright green dots represent callose deposits in epidermal cells). Callose was quantified as described previously (Materials & Methods) (Luna et al. 2011). Values represent percentages of the mean ± SEM.

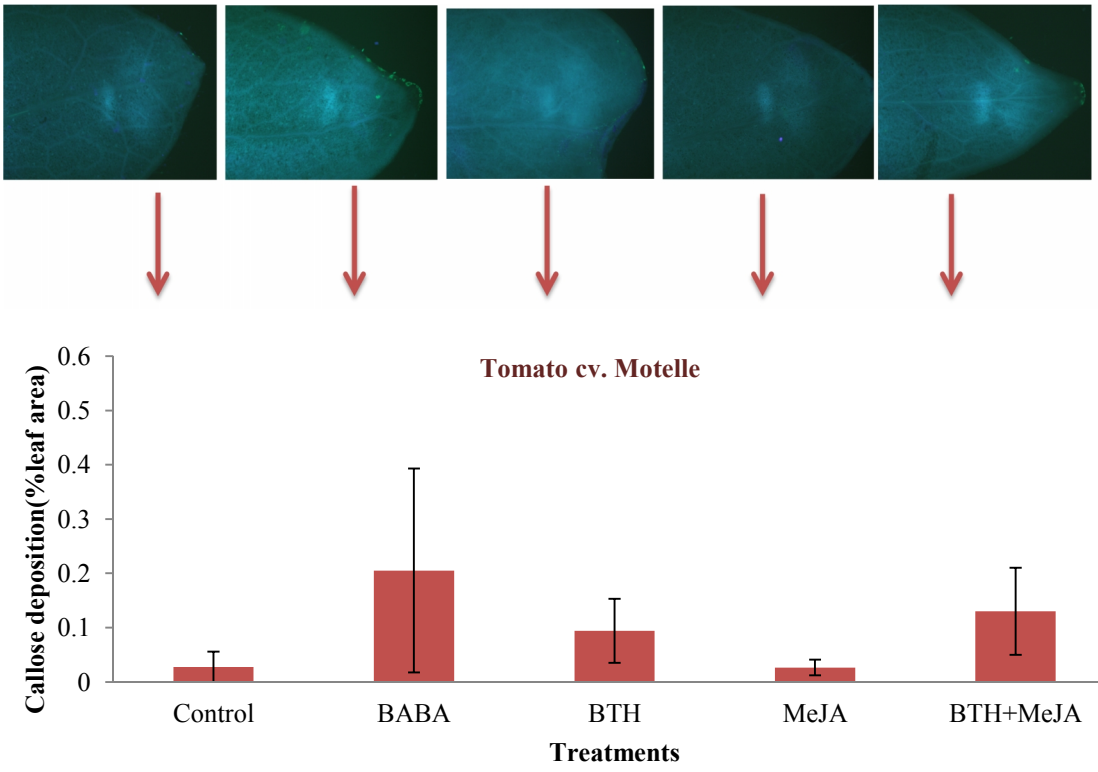


Figure 2.13 Basal callose deposition in tomato cv. Motelle leaves after water-control, BABA, BTH, MeJA and MeJA+BTH treatments. Seven days after treatment leaves were harvested for aniline blue staining. Pictures were taken under fluorescence microscopy at 4x magnifications (bright green dots represent callose deposits in epidermal cells). Callose was quantified as described previously (Materials & Methods) (Luna et al. 2011). Values represent percentages of the mean ± SEM.

To define whether chitosan directly induces callose deposition the experiment was repeated and tomato cv. Moneymaker and cv. Motelle were treated with BABA, MeJA and the 2 chitosan formulations. The trending in both cultivars was similar, with almost no callose induction by any of the elicitors. BABA induced callose apposition was higher in tomato cv. Motelle than in cv. Moneymaker (Figures 2.14 and 2.15); nevertheless, callose induction was generally lower than expected in BABA treatments (as compared with *A. thaliana*, (Ton & Mauch-Mani 2004)). However, the commercial chitosan formulation ChitoPlant highly induced callose apposition in both cultivars in comparison with the rest of the elicitor treatments (Figures 2.14 and 2.15).

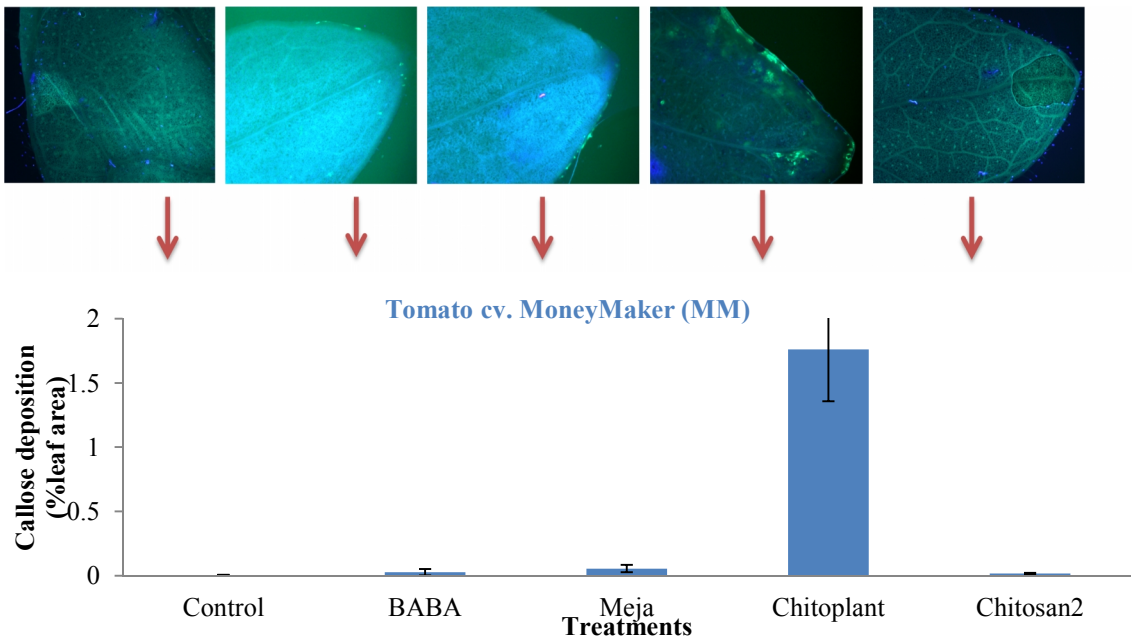


Figure 2.14 Callose deposition in tomato cv. Moneymaker leaves after water-control, BABA, MeJA, ChitoPlant (ChiPro) and Chitosan2 treatments. Seven days after treatment leaves were harvested for aniline blue staining. Pictures were taken under fluorescence microscopy at 4x magnifications (bright green dots represent callose deposits in epidermal cells). Callose was quantified as described previously (Material & Methods) (Luna et al. 2011). Values represent percentages of the mean ± SEM.

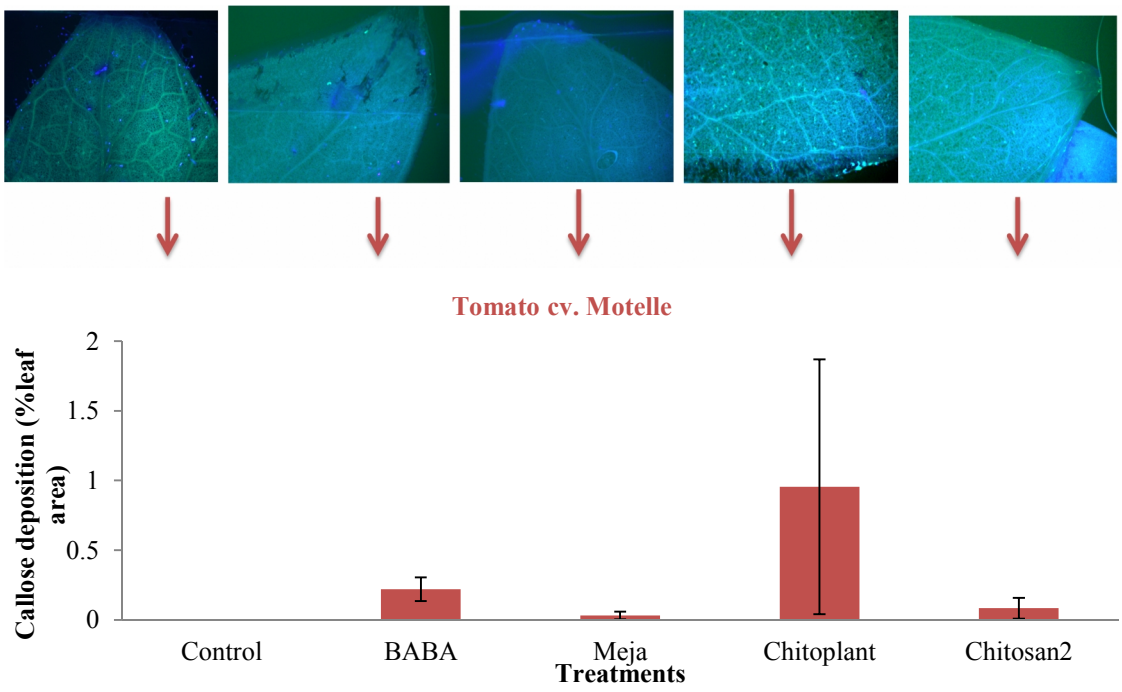


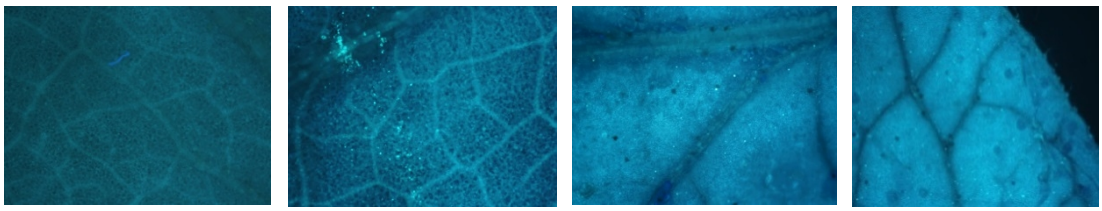
Figure 2.15 Callose deposition in tomato cv. Motelle leaves after water-control, BABA, MeJA, ChitoPlant (ChiPro) and Chitosan2 treatments. Seven days after treatment leaves were harvested for aniline blue staining. Pictures were taken under fluorescence microscopy at 4x magnifications (bright

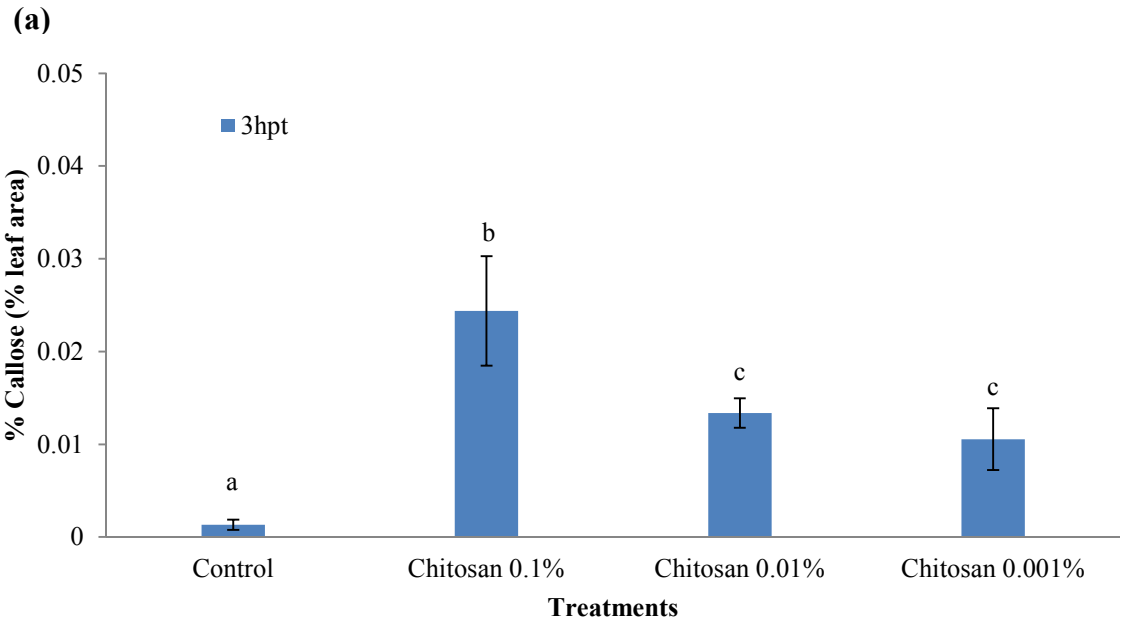
green dots represent callose deposits in epidermal cells). Callose was quantified as described previously (Materials & Methods) (Luna et al. 2011). Values represent percentages of the mean ± SEM.

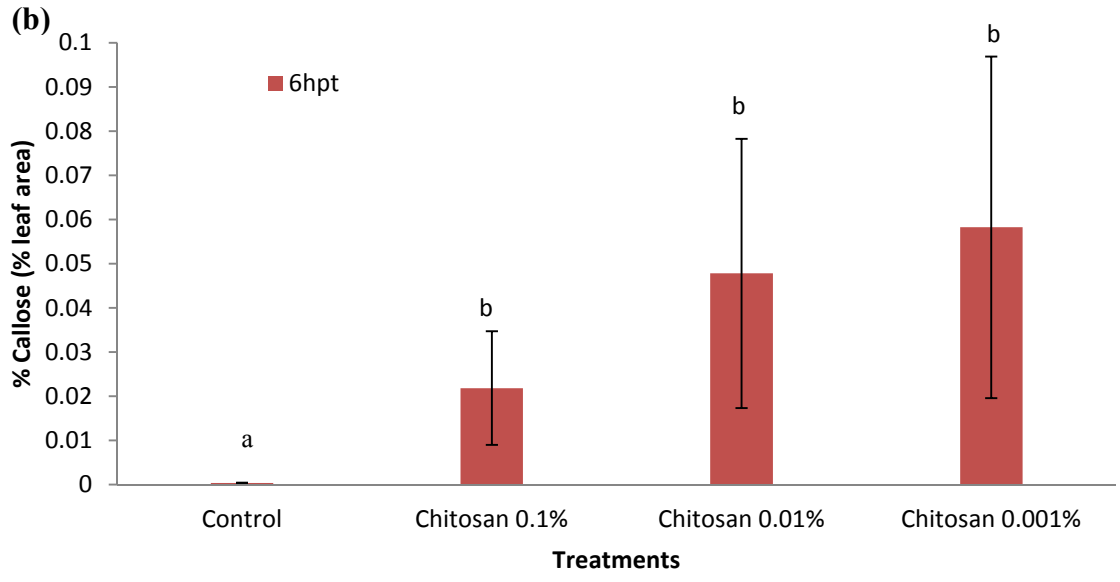
2.3.3.2 Basal callose deposition induced by chitosan in tomato cv. Moneymaker

It has been previously shown that high concentrated ChitoPlant (1% w/v) is able to induce callose deposition in tomato cvs. Moneymaker and Motelle (Figures 2.14 and 2.15). However, high concentrations of chitosan can have a negative effect on plant defences by inducing cell death (2.3.5 Costs of elicitor-induced resistance, Pictures 2.2 and 2.3). Thus, in order to test whether chitosan (ChitoPlant) induces the deposition of callose in *S. lycopersicum* in a concentration-dependent manner, plants were treated with ddH_2O-mock solution and 3 concentrations of chitosan (0.001%, 0.01% and 0.1% w/v). Plant material was collected at 3, 6 hours, 3 and 5 days post-treatment (hpt, dpt), fixed in ethanol and callose deposition was analysed as described in Materials and Methods.

Overall, during the first hours (3 and 6 hpt) and 1 day after chitosan treatment all concentrations resulted in statistically significant callose deposition whilst at later time (5 dpt) none of the concentrations did (P=0.06) in comparison with water/control treated plants (Figure 2.16d), which suggests that chitosan-induced callose decreases gradually after 1 dpt. The lowest concentration (0.001% w/v) significantly induced the highest amount of callose deposits in tomato epidermal cells at 1 dpt (Figure 2.16c), followed by the other 2 concentrations which were significantly higher than water-treated control plants but lower than the low concentration 0.001% (Figure 2.16c).




(a)
0.05
0.04
0.03
0.02
0.01
0
3hpt
% Callose (% leaf area)
a
b
c
c
Control
Chitosan 0.1%
Chitosan 0.01%
Chitosan 0.001%
Treatments


(b)
0.1
0.09
0.08
0.07
0.06
0.05
0.04
0.03
0.02
0.01
0
6hpt
% Callose (% leaf area)
a
b
b
b
Control
Chitosan 0.1%
Chitosan 0.01%
Chitosan 0.001%
Treatments

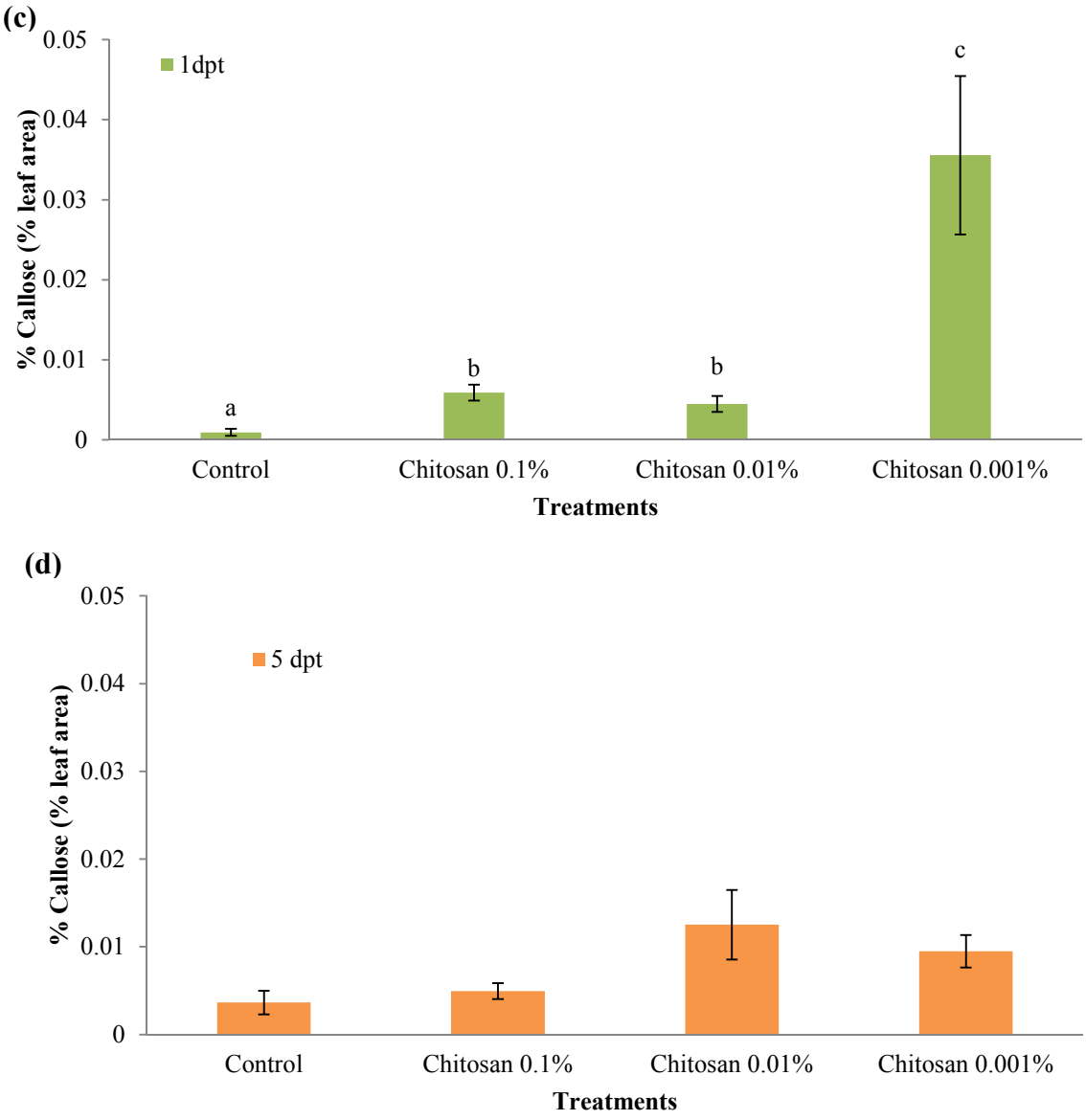


Figure 2.16 Basal callose deposition in tomato cv. Moneymaker leaves after ddH_2O-control and chitosan (ChitoPlant) treatments at 3 different concentrations. 3 and 6 hours, and 1 and 5 days post-treatment leaves were harvested for aniline blue staining (M&Ms). Photographs were taken under fluorescence microscopy (bright green dots represent callose deposits in epidermal cells). Callose was quantified as described by (Luna et al. 2011) (M&Ms). Values represent percentages of the mean ± SEM. Letters indicates statistically significance between the treatment and the water control (t-test $p<0.05$) Kruskal-Wallis test (3 hpt p value $P< 0.001$; 6 hpt p value $P< 0.001$; 1 dpt p value $P< 0.001$; 5 dpt p value $P=0.06$).

Callose induction by ChitoPlant in tomato was concentration-dependent at 3 hpt (Figure 2.16a), where the highest concentration (0.1%) induced more callose sooner and decreased faster than the lower concentrations. However, it did not follow the same pattern at 6 hpt and 1 dpt and 5 dpt (Figure 2.16b, 2.16c, 2.16d), where the lowest concentrations (0.01% and 0.001%) significantly induced callose deposition for a longer

period (1 dpt) and more than the highest concentration (0.1%), indicating that elicitor-priming does not follow a classical dose-response curve and the duration of the response is inversely affected by concentration. However, this duration may be related to chitosan concentration.

2.3.3.3 Basal callose deposition induced by chitosan in *Arabidopsis thaliana*

A similar assay was conducted to test whether chitosan (Chitoplant) induces the deposition of callose in a different species and in a concentration-dependent manner. *A. thaliana* Col-0 (wild-type) plants were treated with distilled water (control), 0.01%, 0.1% and 1% w/v of chitosan by spraying the solution onto the plants. Plant leaves were harvested at 1 dpt and callose was quantified (Materials & Methods).

Chitosan was able to induce callose deposition in Arabidopsis but the amount varied depending on the concentration, being significantly different from water-control treatment only at 0.01% w/v one day post-treatment (Figure 2.17).

High concentration (1% w/v) of chitosan induced large amount/pieces of callose deposits, which can ultimately damage the plant. This effect lead to a big variability and affected statistical significance. Thus, although not significantly different from the other concentrations, chitosan 1% w/v appeared to induce the highest concentration of callose followed by chitosan 0.01% w/v (the lowest concentration) (Figure 2.17), which suggests that there is no clear dose-response curve for such a priming agent.

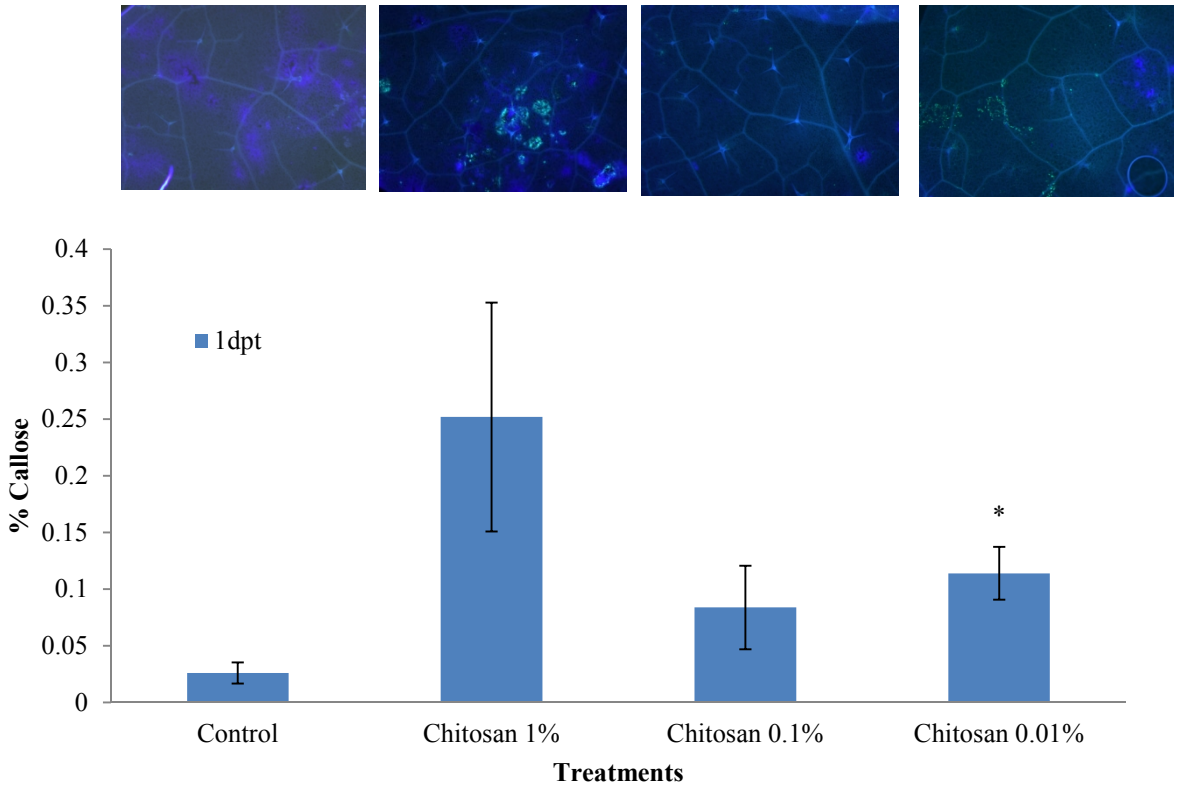


Figure 2.17 Basal callose deposition in *Arabidopsis thaliana* Col-0 leaves after ddH_2O-control and chitosan treatments at 3 different concentrations. 1 day after treatment leaves were harvested for aniline blue staining. Pictures were taken under fluorescence microscopy. Callose was quantified as described by (Luna et al., 2011) (M&Ms). Values represent percentages of the mean ± SEM. Asterisk indicates statistically significance between the treatment and the water control (t-test $p<0.05$ Kruskal-Wallis test (1 dpt $p=0.02$)).

2.3.3.4 Pathogen-induced callose deposition in BABA, MeJA, BTH+MeJA, two chitosan formulations and BTH in tomato cv. Moneymaker and cv. Motelle

It has been previously shown that MeJA, BABA, BTH, Chitosan2 and ChitoPlant can significantly induce resistance in tomato cvs. Moneymaker and Motelle against *B. cinerea* (Figures 2.1 and 2.2). Furthermore, it was discovered that MeJA (Figure 2.12) and mainly chitosan (ChitoPlant formulation) can induce callose deposition (Figures 2.14 and 2.15) in both tomato cultivars in the absence of pathogen.

2.3.3.4.1 MeJA primes callose deposition in tomato cv. Moneymaker against aggressive *Botrytis cinerea* strain

To determine the effectiveness of BABA, MeJA, BTH+MeJA, two chitosan formulations and BTH treatments priming cell-wall defences by inducing callose against *B. cinerea*, tomato cvs. Moneymaker and Motelle seedlings were foliar sprayed, 17 days previous infection (long-lasting defence induction), with ddH_2O, BTH, MeJA, a combination of BTH+MeJA and BABA (soil drenched) and subsequently infected with *B. cinerea* as described in Material and Methods. Three days post inoculation double staining (aniline blue + calcofluor, Materials and Methods) was performed in order to see pathogen-induced callose deposition in all treatments.

Despite the high level of aggressiveness of the *B. cinerea* R16 strain (strain used during all experiments) and the high contrast of the calcofluor, which made it difficult to measure callose deposition by microscopy, callose was found in tomato cv. Moneymaker samples treated with MeJA (Figure 2.18), which correlates with the significant lesion size reduction of the same treatment (Figure 2.1 and 2.2). Callose was not seen in the rest of the elicitor-treated plants or in the water-treated control plants (Figure 2.18).

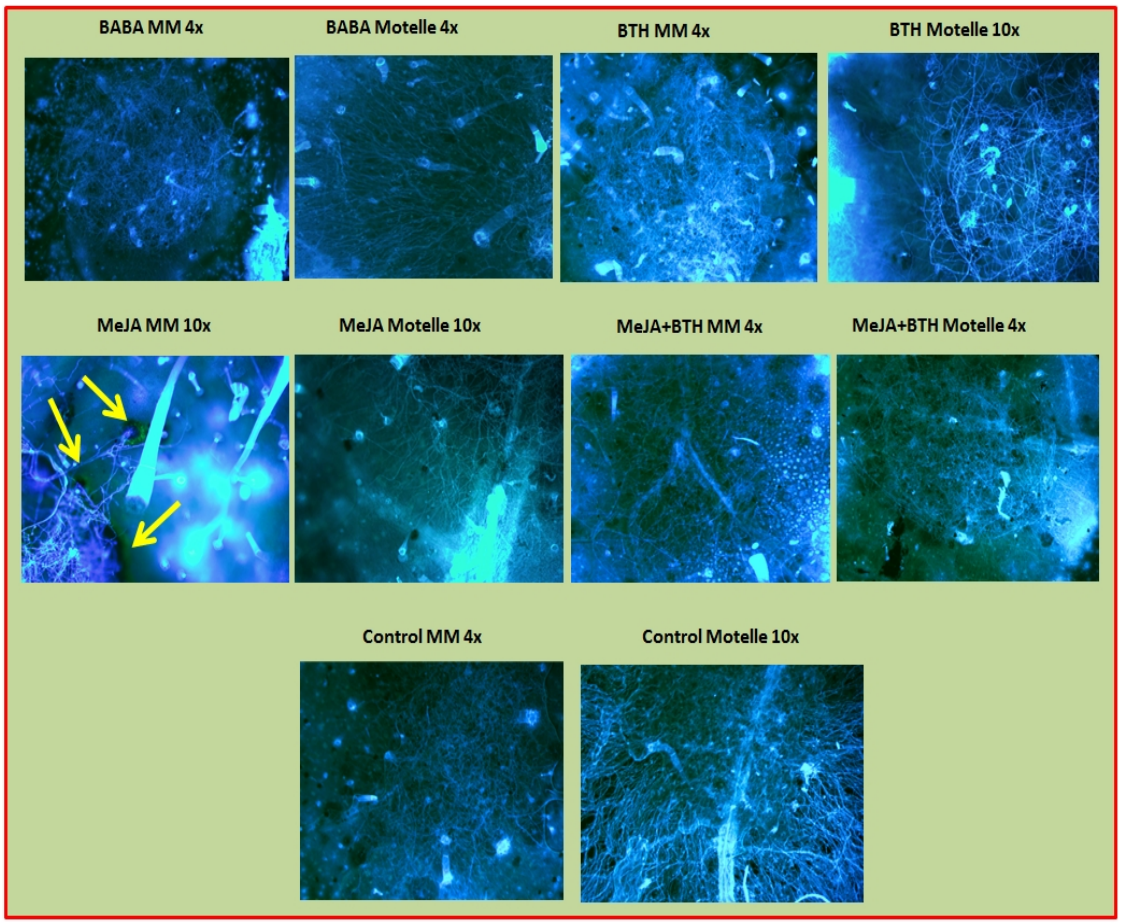


Figure 2.18 Callose deposition (bright green dots highlighted with yellow arrows represent callose deposits in tomato epidermal cells close to fungal penetration sites) in cv. Moneymaker and cv. Motelle leaves after *B. cinerea* infection in water-control, BABA, BTH, MeJA and MeJA+BTH-treated plants. 3 days post inoculation, leaves were harvested for double staining (aniline blue + calcofluor) and then pictures were taken under fluorescence microscopy at 4x and 10x magnifications.

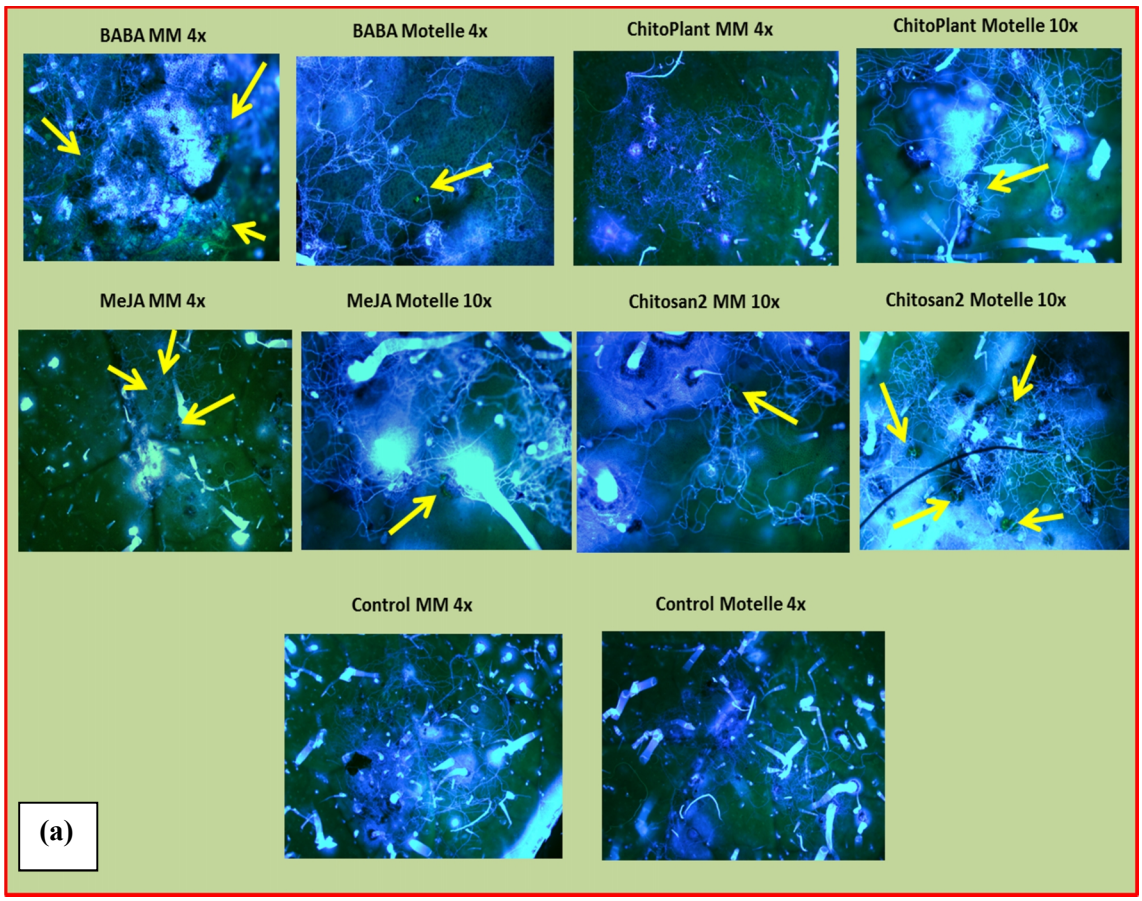
In cv. Motelle leaves, callose was not seen in any of the elicitor-treated plants, results that correlate with the generally higher susceptibility of the cv. Motelle to *B. cinerea* (Figure 2.3).

2.3.3.4.2 MeJA, BABA and chitosan can prime callose deposition in tomato cvs. Moneymaker and Motelle against less virulent *Botrytis cinerea*

Another similar experiment was conducted in order to determine the effectiveness of chitosan treatment priming cell-wall defences against less virulent *B. cinerea* infection (adjusted concentration). Tomato cvs. Moneymaker and Motelle seedlings were foliar sprayed, 17 days previous infection (long-lasting defence induction), with ddH_2O, BABA (soil drenched), Chitosan2, ChitoPlant and MeJA and subsequently infected with *B. cinerea* as described in Materials and Methods. Three days post-inoculation

double staining (aniline blue + calcofluor, Materials and Methods) was performed in order to see pathogen-induced callose deposition in all treatments.

Due to the less concentrated pathogen spore inoculum (adjusted to 2 x 10^4 spores/ mL), more differences were seen among treatments. In general, lesions in BABA-treated plants were smaller and appeared to have less mycelia. Water-treated control plants did not show callose deposition in any of the samples harvested (Figure 2.19a). As previously seen (Figure 2.18), in cv. Moneymaker callose papillae formation was greater than cv. Motelle and callose was accumulated surrounding some parts of the inoculum droplet, which presumably slowed down *B. cinerea* expansion (Figure 2.19). MeJA-treated plants also produced callose around the penetration sites of the hypha with no differences between both cultivars. ChitoPlant-treated plants deposited low amount of callose in comparison with the mock treatments in cv. Moneymaker (Figures 2.14 and 2.15). In contrast, Chitosan2-treated cv. Motelle plants showed greater callose apposition surrounding hypha penetration sites. Due to these unexpected results further experiments need to be done in order to see consistency or variance in BABA, MeJA and chitosan-induced callose deposition after pathogen challenge.



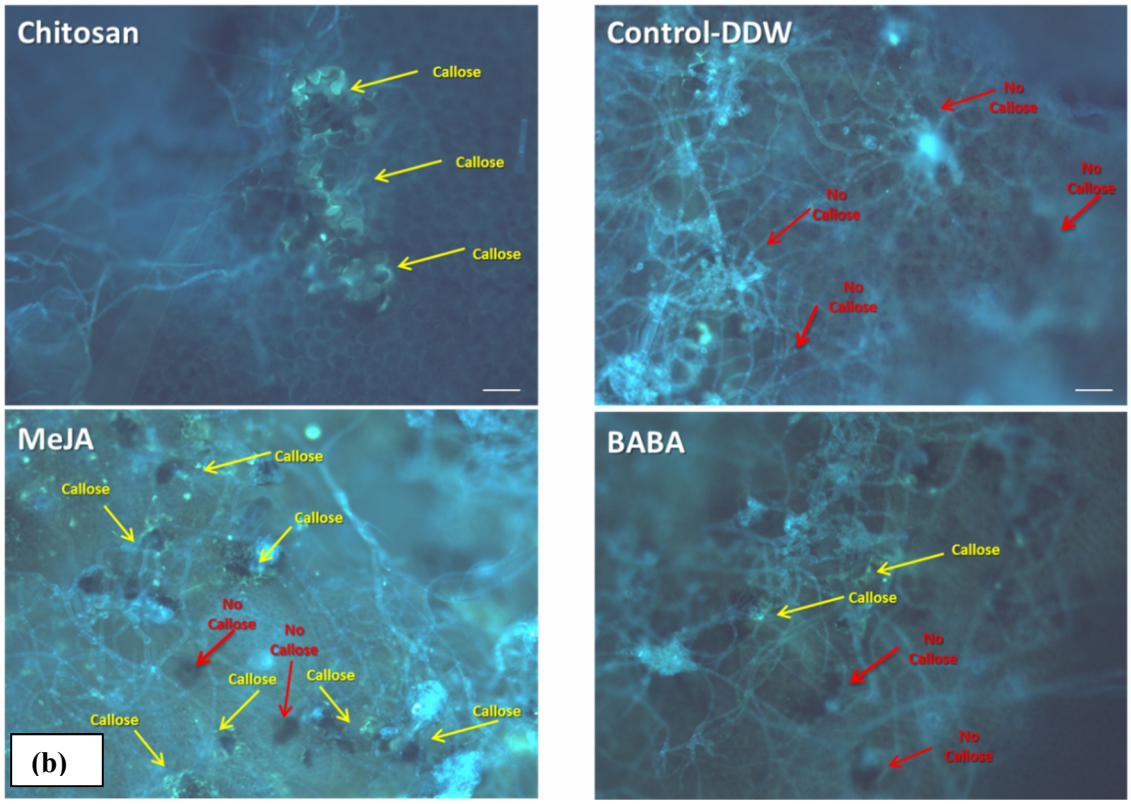


Figure 2.19 **(a)** Callose deposition (bright green dots highlighted with yellow arrows represent callose deposits in tomato epidermal cells close to fungal penetration sites) in tomato cv. Moneymaker and tomato cv. Motelle leaves after *B. cinerea* infection in ddH_2O (SDW)-control, BABA, MeJA, ChitoPlant and Chitosan2-treated plants. 2 dpi leaves were harvested for double staining (aniline blue + calcofluor) and then pictures were taken under fluorescence microscopy at 4x and 10x magnifications. **(b)** Callose deposition in tomato cv. Moneymaker leaves after *B. cinerea* infection in ddH_2O-control, BABA (as a positive control), MeJA and chitosan treated plants. Three days post-inoculation leaves were harvested for double staining (aniline blue + calcofluor) and then pictures were taken under fluorescence microscopy at 4x and 10x magnifications.

Furthermore, MeJA-treated plants were able to highly induce callose around most of the penetration sites of the hyphae as well as outside of *B. cinerea* penetration sites (Figure 2.19b). ChitoPlant-treated plants also induced callose in a lesser extent; however it was more localized to *B. cinerea* penetration sites (Figure 2.19b). BABA-treated plants rarely induced callose deposition towards *B. cinerea* infection sites.

2.3.3.5 Chitosan and MeJA peroxidase (POD) activity and H_2O_2 production induction against *Botrytis cinerea*

So far, it has been shown how two resistance elicitors, MeJA and chitosan, are involved in cell-wall defences through callose deposition. However, it is well-known that reactive oxygen species (ROS) accumulation is also part of plant initial defence response after

pathogen challenge (Mehari et al. 2015). Thus, to elucidate whether chitosan and MeJA are involved in ROS production in tomato against *B. cinerea* the effects of chitosan (ChitoPlant formulation), MeJA and the combination of chitosan + MeJA on increasing peroxidase activity in tomato cv. Moneymaker and tomato *sitiens* (resistant mutant tomato line, Materials and Methods) after *B. cinerea* infection was investigated. For this, tomato plants were foliar sprayed with the elicitors and 4 days after treatment and leaf tissue discs were harvested for extracellular peroxidase activity evaluation with the tetramethylbenzoidine (TMB) assay, as described in Materials & Methods.

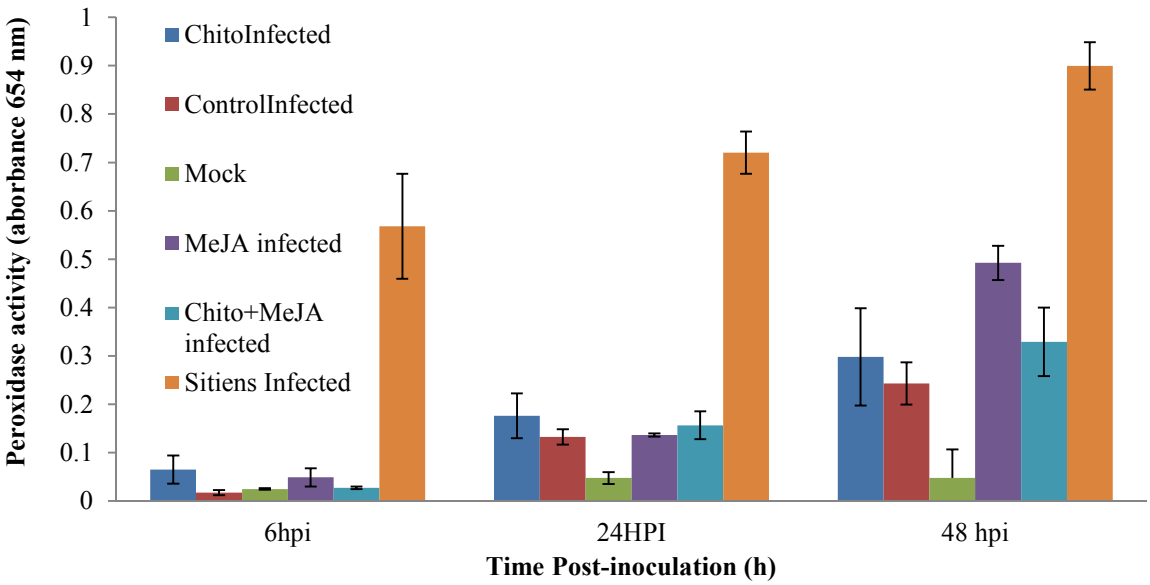


Figure 2.20 Extracellular peroxidase activity in tomato susceptible cv. Moneymaker (wild-type) (treated with MeJA, chitosan, MeJA+chitosan and water/control) and untreated tomato ABA deficient-Sitiens (resistant mutant line to *Botrytis cinerea*) leaf discs infected with *B. cinerea*. Tissue sampling was done at 24 and 48 hours post infection (hpi). Values presented are means of 3 biological replicates ± SEM.

Extracellular peroxidase activity assay showed that, as *B. cinerea* infection progresses, production of peroxidases are enhanced. The resistant tomato mutant *sitiens* (orange bars) showed highly increased POD activity at all time points after infection (Figure 2.20). For the rest of treatments, in susceptible wild-type accession (tomato cv. Moneymaker) chitosan and MeJA elicitors appeared to increase POD activity at all time points but these differences were not significant. Only chitosan-treated wild-type plants were able to induce POD activity at early stages of the infection (6 hpi) (Figure 2.20). MeJA-treated wild-type plants were able to increase POD production at 48 hpi (when the necrotic lesions are visible) in comparison with control infected plants which

suggests the potential ability of chitosan to prime tomato peroxidases at early stages of the infection, while MeJA may prime tomato peroxidases at later stages against *B. cinerea*. However, the combination treatment chitosan + MeJA did not have a synergistic effect on extracellular peroxidase expression against *B. cinerea* at any time point.

Production of H_2O_2 can result from increased peroxidase activity and peroxidases mediate many H_2O_2-related defence responses (Asselbergh et al. 2007a). Thus, in order to investigate chitosan and MeJA roles in temporal evolution of H_2O_2 accumulation after pathogen challenge, tomato cv. Moneymaker plants were foliar sprayed, 4 days before *B. cinerea* infection, with ddH_2O, chitosan (ChitoPlant) 1% w/v and MeJA (0.1 mM) and infected leaves were subsequently stained at 24 and 48 hpi using 3,3'-diaminobenzidine (DAB) as described in Materials and Methods.

Visual analysis revealed that in MeJA-treated and, in a lesser extent, chitosan-treated leaves, H_2O_2 was contained around the local site of infection at 24 hpi and 48 hpi; however in water-treated (control) leaves H_2O_2 was not restricted at the infection sites and spread throughout a bigger area of the leave (Figure 2.21), suggesting a possible manipulation of *B. cinerea* host defences as stated before (El Oirdi et al. 2011). However, in order to test this theory, further experiments (more time points, different chitosan concentrations and quantitative methodology) need to be done.

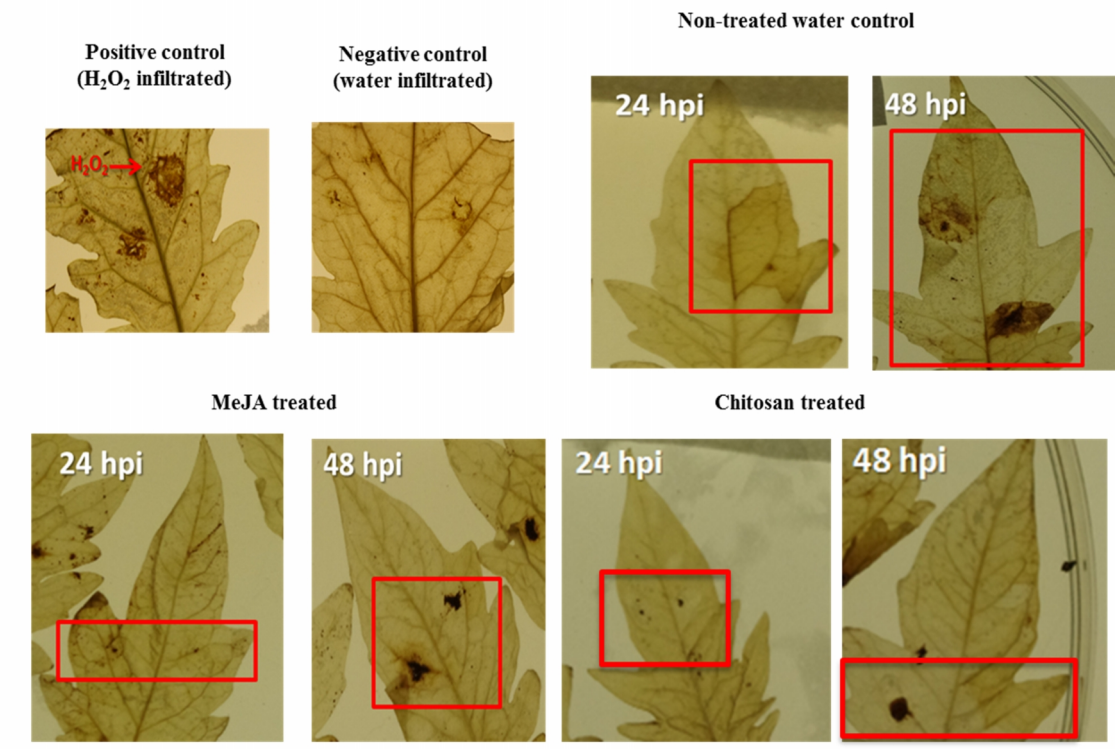


Figure 2.21 Temporal evolution of H_2O_2 accumulation (brown dark dots) in tomato cv. Moneymaker sprayed with either 100 µM MeJA, 1% w/v chitosan and ddH_2O as control before infection with *B. cinerea*. DAB staining of detached leaves infected with two 6 µL drops of a spore suspension was performed at different time points after *B. cinerea* infection (24 and 48 hpi). One representative leaflet of three replicates is shown for each time point. Red boxes represent infection site and H_2O_2 area.

Thus, MeJA and chitosan might reduce and limit pathogen infection through localizing H_2O_2 production to the infection site (Figure 2.21), potentially reducing pathogen manipulation of its host defences.

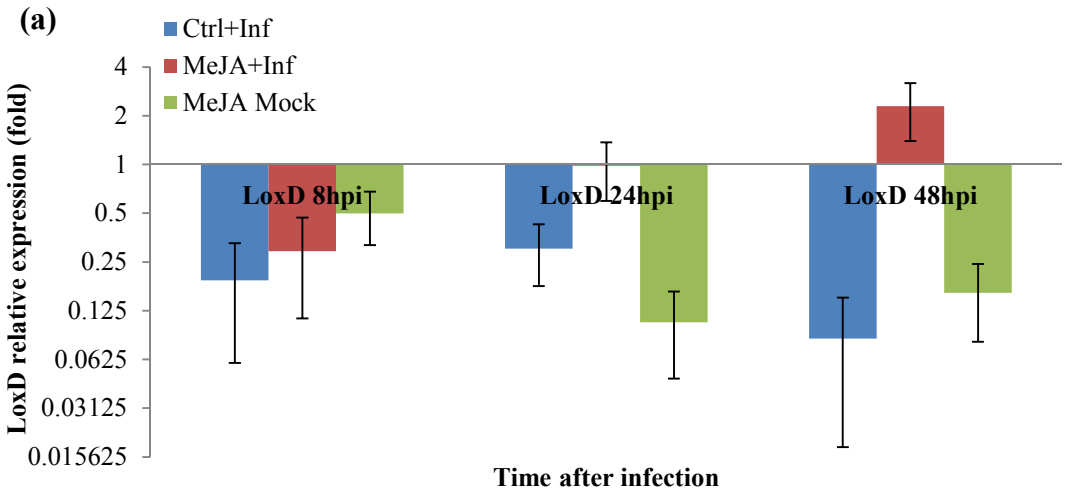
2.3.4 Hormone-related defence gene expression in *Solanum lycopersicum*

2.3.4.1 MeJA-priming of defence marker genes in tomato against *Botrytis cinerea*

To determine whether the phytohormone derivative methyl-jasmonate (MeJA) acts through priming of gene expression of marker genes, gene expression analysis of two tomato defence genes, leucine aminopeptidase (Lap) A, a gene involved the plant-defence response against mechanical wounding, insect infestation, and in response to pathogen infection (Pautot et al. 2001); and lipoxygenase (Lox) D, a gene involved in jasmonic acid defences and biosynthesis (Scranton et al. 2013), the main defence phytohormone against necrotrophic pathogens, such as *B. cinerea*, was investigated.

Five days after tomato cv. Moneymaker plants were treated with MeJA (0.01 mM) and ddH_2O as a control treatment, they were infected with *B. cinerea* spore inoculum (Materials & Methods) and mock inoculated (ddH_2O) as a non-infected control. 8, 24 and 48 hour post-infection (hpi) tissue samples were collected. qPCR analysis shows that LapA and LoxD transcripts expression were repressed by the infected treatments (MeJA+Inf and Ctrl+Inf) and by MeJA without infection (MeJA Mock) at 8 hpi (Figure 2.22a). However, LoxD transcript was not down-regulated at 24 hpi and up-regulated at 48 hpi only by MeJA+ infected plants whilst MeJA mock plants and water-treated (control, Ctrl) + infected plants did not induce it. In contrast, LapA transcript levels were up-regulated by MeJA without the pathogen and both infected treatments were not able to induce it at 24 hpi and 48 hpi (Figure 2.22b). MeJA mock plants were the only treatment that kept LapA expressed after 48 hpi while it was down-regulated by the infection treatments, being MeJA able to reduce LapA repression by the pathogen at both last time points (Figure 2.22b).

To test whether *B. cinerea* is able to manipulate the antagonistic cross-talk between JA and SA pathways through NPR1 (El Oirdi et al. 2011), NPR1 expression was tested. NPR1 expression did not significantly differ among treatments although there was a putative effect of MeJA-infected plants were NPR1 was repressed at 8 hpi but not in control-infected (Ctrl + Inf) plants, whereas it was not induced nor repressed by MeJA + infection at 24 and 48 hpi (Figure 2.22c).



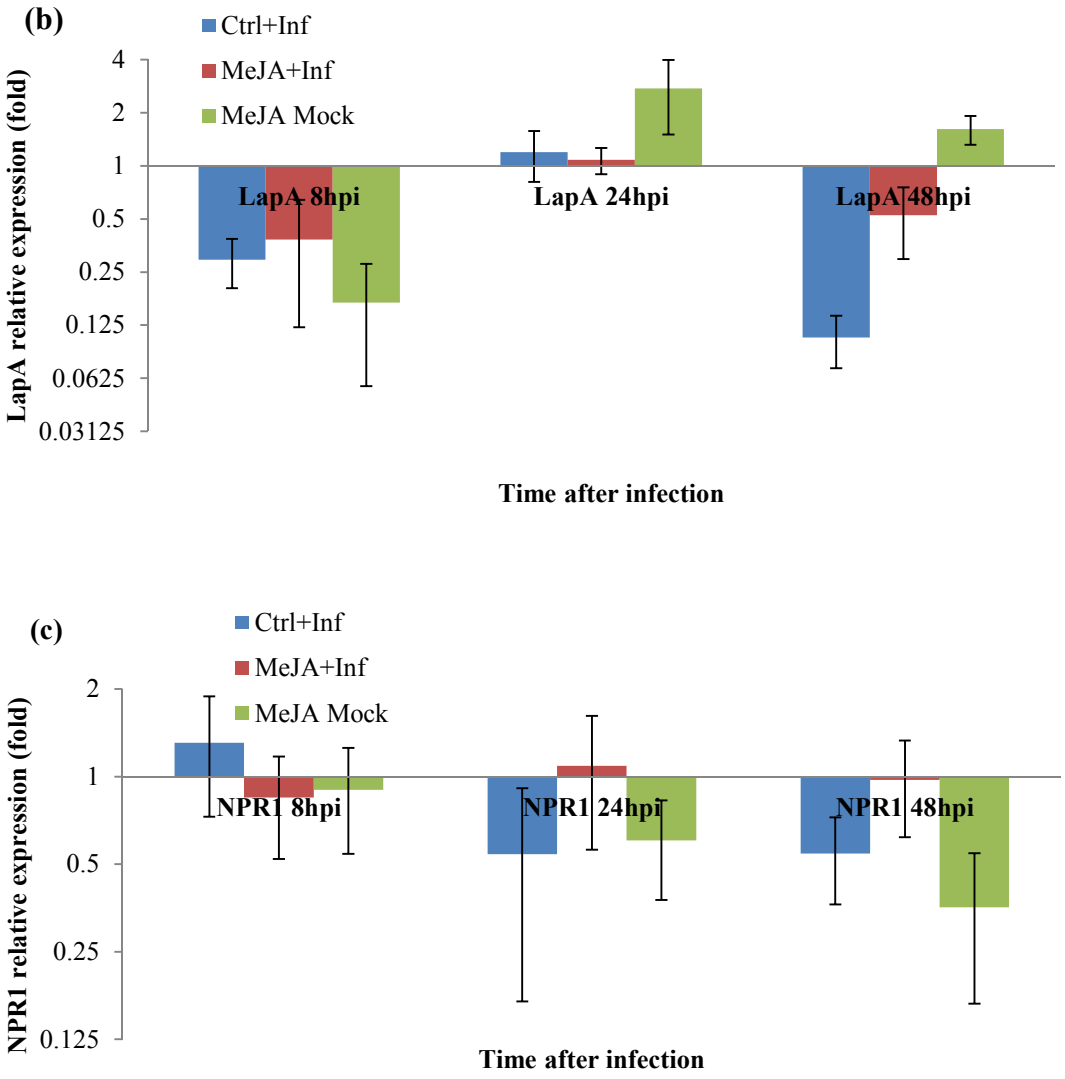


Figure 2.22 **(a)** LoxD, **(b)** LapA and **(c)** NPR1 relative expression (fold change, Log2). Total RNA was extracted, and the levels of LoxD, LapA and NPR1 were determined by qRT-PCR. Ctrl+Inf, untreated and *B. cinerea*-infected plants; MeJA, MeJA-treated and non-infected plants, MeJA+Inf, MeJA-treated and infected plants. EF1-α served as internal reference. The data shown belongs to the relative gene expression to the control-water mock treatment, means of three biological replicates for the qRT-PCR ± standard error of the mean (SEM).

2.3.4.2 Elicitor direct induction of SA & JA-defence genes in tomato against *Botrytis cinerea*

As seen in the previous experiment, MeJA is able to prime jasmonic acid (JA)-dependent LoxD (Figure 2.22) transcript against *B. cinerea*. Jasmonic acid and salycilic acid pathways can be mutually antagonistic (Mur et al. 2006). Moreover, it has been previously shown that *B. cinerea* is able to manipulate both phytohormone pathways to promote disease in tomato through Nonexpressed Pathogen Related1 (NPR1) (El Oirdi et al. 2011), which makes these signalling pathways important in the ultimate outcome of tomato-*B.cinerea* interaction.

Thus, to evaluate the contribution of candidate elicitors BTH, BABA, MeJA, SoftGuard (this chitosan + chitin commercial elicitor was used in replacement of the previous formulations due to an issue/time with the supply) in the expression profile of two tomato marker defence genes belonging to both jasmonic (JA) and salycilic acid pathways respectively (PI I or Pin1 and PR-1), tomato cv. Moneymaker plants were foliar sprayed with ddH_2O (control), BTH, MeJA, BABA and SoftGuard and leaf tissue was harvested at 3, 9 and 24 hours after treatment for total RNA extraction. Subsequent qRT-PCR was performed to test gene expression of PR1 and Pin1 (PI I) (M&Ms).

qRT-PCR analysis revealed that tomato SA-dependent PR-1 was highly induced at 3 hours after treatment (hat) of BABA treatment in comparison with the other treatments (Figure 2.23). BTH was able to induce PR-1 expression in an increased manner over the time. MeJA induced PR-1 expression at 3 and 9 hat, however MeJA induction of PR-1 was reduced at 24 hat compared to non-treatment (control) (Figure 2.23). In contrast, the combination of chitin + chitosan elicitor (Softguard) was only able to down-regulate PR-1 at 3 hat and did not induce PR-1 at 9 and 24 hat.

JA-dependent tomato proteinase inhibitor I (PI I/Pin1) was quickly induced at 3 hat after MeJA and BABA treatments in comparison with non-treatment (control) (Figure 2.23). However, BABA did not have an effect on PI-I expression later at 9 and 24 hat. The JA-positive control methyl jasmonate (MeJA) highly induced PI-I at all time points, whereas SoftGuard and BTH were only able to induce PI-I at 9 hat (Figure 2.23).

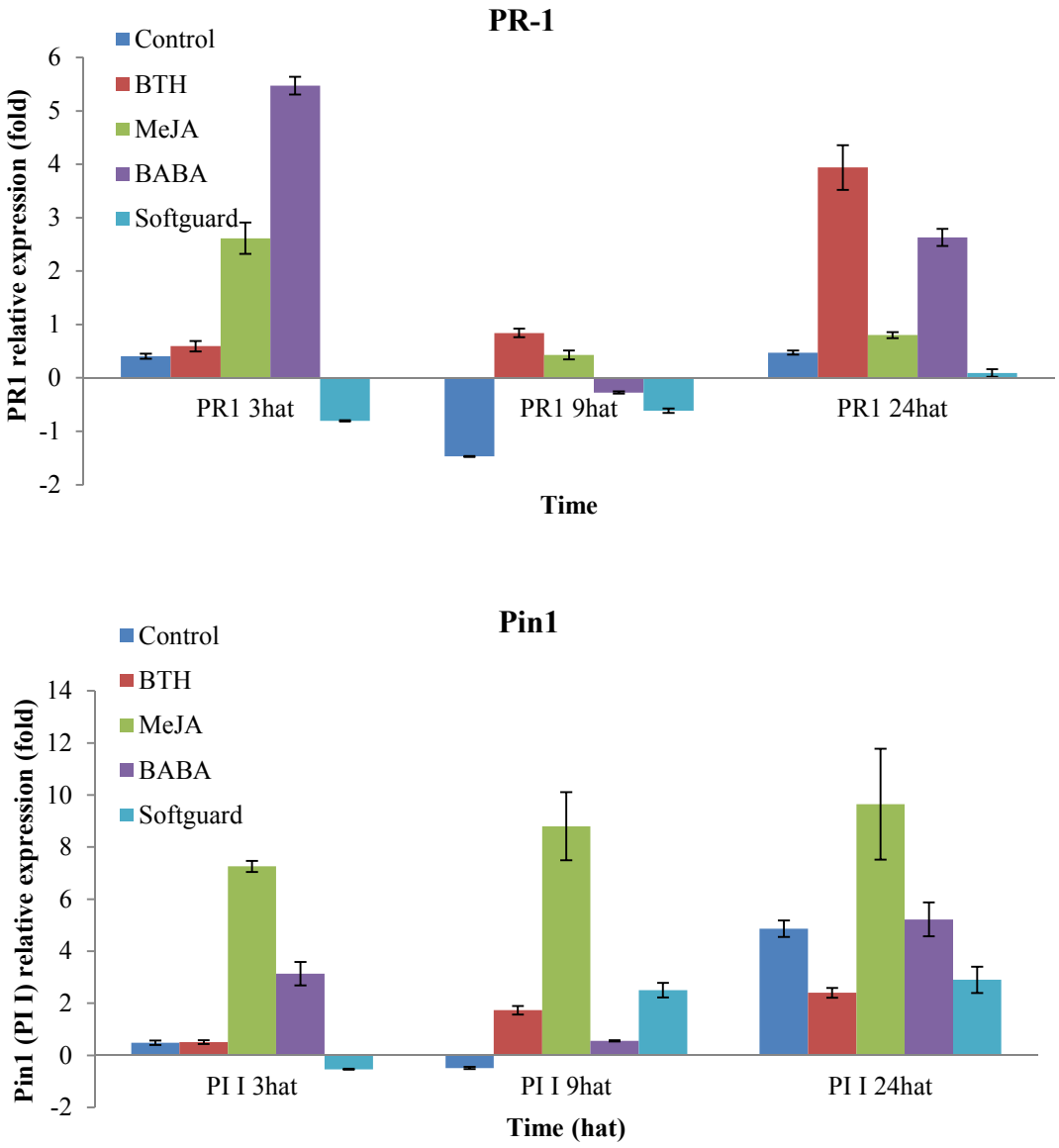


Figure 2.23 Expression levels of tomato SA-dependent PR-1 and JA- protein inhibitor Pin1 (also PI-I) relative to Actin (reference gene). Fold change, Log2. Four-week-old plants were treated with BTH, MeJA, BABA, Softguard (chitin+chitosan) and water (control); Samples (leaves) were harvested at 3 time points (3h, 9h and 24 hours after treatment) for RNA extraction. qRT-PCR was performed with specific primers for tomato PR-1, Pin1 (PI I) and Actin (reference control gene) as described in Methods. Values represent means relative to Actin and 3 hours control treatment ±SD from three biological replicates.

2.3.5 Costs of elicitor-induced resistance in tomato development and cell death

Elicitor-induced resistance on mainly tomato and potentially on other plants is the core of this Ph.D project and therefore, to better understand how elicitor may affect the crop after treatment, and to achieve a more efficient induced resistance with minimal impact

on plant fitness, it is crucial to evaluate the potential costs in plant development caused by elicitor interaction with the plant.

2.3.5.1 Elicitor-induced growth reduction in tomato cvs. Moneymaker and Motelle

To evaluate the costs of the elicitors Chitosan1 (ChitoPlant), Chitosan2, methyl-jasmonate (MeJA), Benzo (1,2,3)-thiadiazole-7-carbothioic acid S- methyl ester (BTH, BION), the combination of MeJA + BTH and β-amino-butyric acid (BABA) treatments in plant development, tomato cvs. Moneymaker and Motelle plants were foliar treated with these elicitors and plant height was measured (M&Ms).

In 1- to 2 -week-old plants, defence induction by BABA and MeJA lead to statistically significant reductions in relative growth rate (RGR) (see M&Ms) in both cultivars (Figure 2.24). Although there were not significant differences between both cultivar responses to the elicitors, there was a trending of a more susceptibility towards BABA-induced stress in cv. Motelle than cv. Moneymaker, since BABA reduced the RGR in cv. Moneymaker by 39% and by a 42% in cv. Motelle. MeJA reduced RGR by 7% in cv. Moneymaker and 15% in cv. Motelle (Fig. 2.24, Table 2.1).

In both cultivars, RGR was significantly higher (Figure 2.24, $P<0.001$) after the induction by BTH and the combination of MeJA + BTH in comparison with water-treated control plants, suggesting that BTH increases plant growth. However, plants developed thinner and smaller stems and fewer secondary leaves (data not shown), thus indicating that BTH does not affect plant height but it does alter normal plant development.

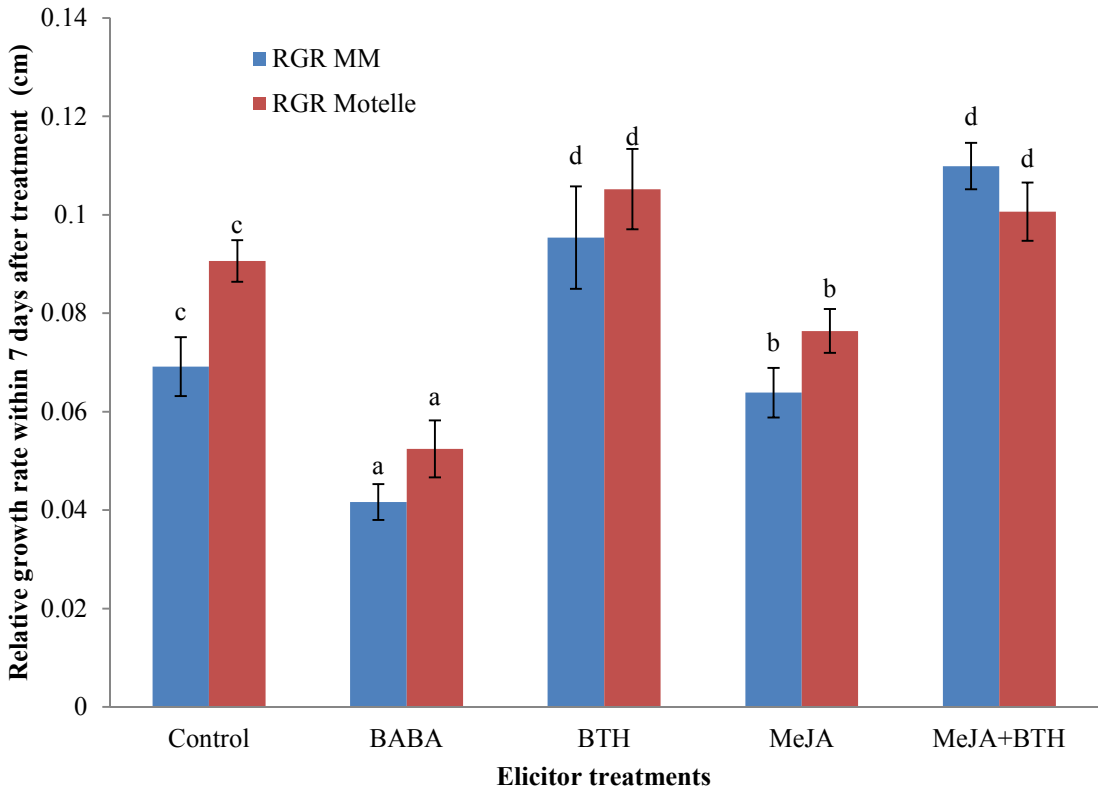


Figure 2.24 Relative growth rate of tomato cvs. Moneymaker (MM) and cv. Motelle after elicitor and water (control) treatment during seven days after treatment. Values presented are means ± SEM. Different letters indicate statistically significant differences (Fisher's least significant difference (LSD) test $P<0.001$, $\alpha=0.05$) between every elicitor treatment compared to water-control (Pairwise test independent per tomato cultivar).

% Repression in growth		
TREATMENT	**% Moneymaker**	**% Motelle**
BABA	39.7	42.1
MeJA	7.6	15.7
BTH	-37.9	-16.1
MeJA + BTH	-58.9	-11

Table 2.1 Percentage of repression of growth of tomato cvs. Moneymaker and cv. Motelle after elicitor treatment seven days after treatment. Values are the percentage of the relative growth rate (RGR) of the elicitor divided by water-control RGR.

Picture 2.1. Two-week-old tomato cvs. Motelle (red) and Money-maker (blue) seedlings 1 week after elicitor treatment. BABA-treated plants were smaller than the rest of the treatments and cv. Motelle-BABA treated plants were even smaller than cv. Moneymaker BABA-treated seedlings.

The costs of Chitosan2 and Chitosan1 on plant fitness, such as seedling growth were also analysed by relative growth rate (RGR). Defence induction by BABA lead again to statistically significant reductions in RGR in both cultivars (Figure 2.25a). In contrast, Chitosan1 and MeJA RGR did not significantly differ from the control plants. MeJA-induced rowth reduction was only obserbed once (Figure 2.24), which could be due to a difference in the absorption efficiency of tomato leaves of the elicitor solution. Chitosan2 RGR was significantly higher than the water-control plants (Figure 2.25a) although plants under this treatment had thinner stems and less secondary leaves development (data not shown). Moreover, as the trending indicated in the previous RGR analysis, this time BABA-induced stress was significantly higher in cv. Motelle than cv. Moneymaker (Figure 2.25b). BABA-growth repression was 32% in cv. Moneymaker (MM) and 45 % in cv. Motelle, suggesting again that cv. Motelle is more susceptible to BABA (Table 2.2).

% Repression in growth		
TREATMENT	**% Moneymaker**	**% Motelle**
BABA	32.6	45.8
MeJA	-14.1	-8.5
Chitosan2	-15.5	-22.9
ChitoPlant (Chitosan1)	-27	-5.7

Table 2.2. Percentage of repression of growth of tomato cvs. Moneymaker (MM) and Motelle after elicitor treatment seven days after treatment. Values are the percentage of the relative growth rate (RGR) of the elicitor divided by water-control RGR.

Thus, it can be observed that some of the elicitors, such as BABA, reduce plant growth in both cultivars (Picture 2.1). However, it seems that the magnitude of the growth suppression is cultivar-dependent, as the tomato cv. Moneymaker shows less BABA-induced growth reduction (Figure 2.25ab and Table 2.2). This relative-growth rate analysis helped to understand how crop cultivars can be affected differently to the stimulus, verifying that every cultivar responded in a different manner to elicitor-induced fitness costs for all elicitors except for Chitosan1 (ChitoPlant), which had a similar effect on the RGR of both cultivars (Figure 2.25b).

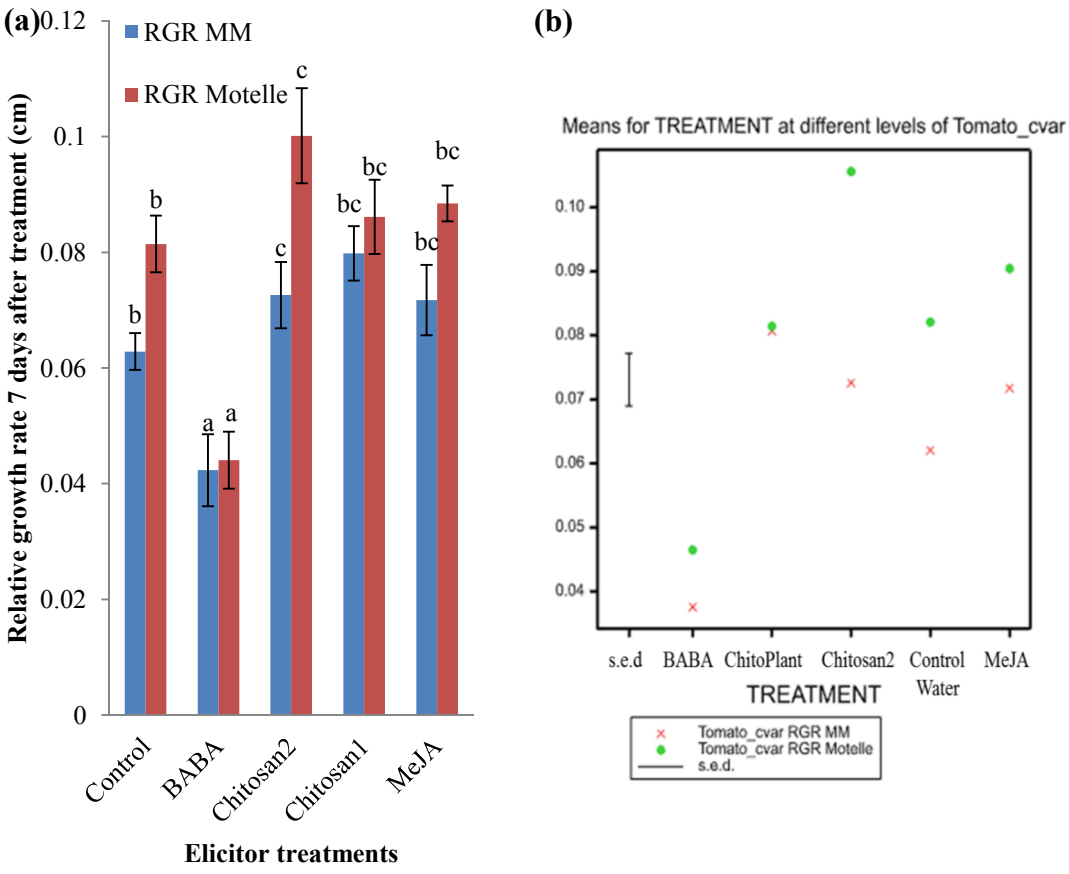


Figure 2.25 (**a**). Quantification of relative growth rate of 2 tomato cultivars after elicitor and water (control) treatment, during 7 consecutive days. Values presented are means ± SEM. Different letters indicate statistically significant differences (Fisher's least significant difference (LSD) test $P<0.001$, $\alpha=0.05$) between every elicitor treatment compared to water-control (Pairwise test independent per tomato cultivars). (**b**). Quantification of relative growth rate of 2 tomato cultivars after elicitor treatment, during 7 consecutive days. Values represented are means (of the RGR in cm) ± SEM obtained from an ANOVA mean plot ($P<0.001$ for cultivar).

2.3.5.2 Chitosan-induced cytotoxicity in tomato and aubergine

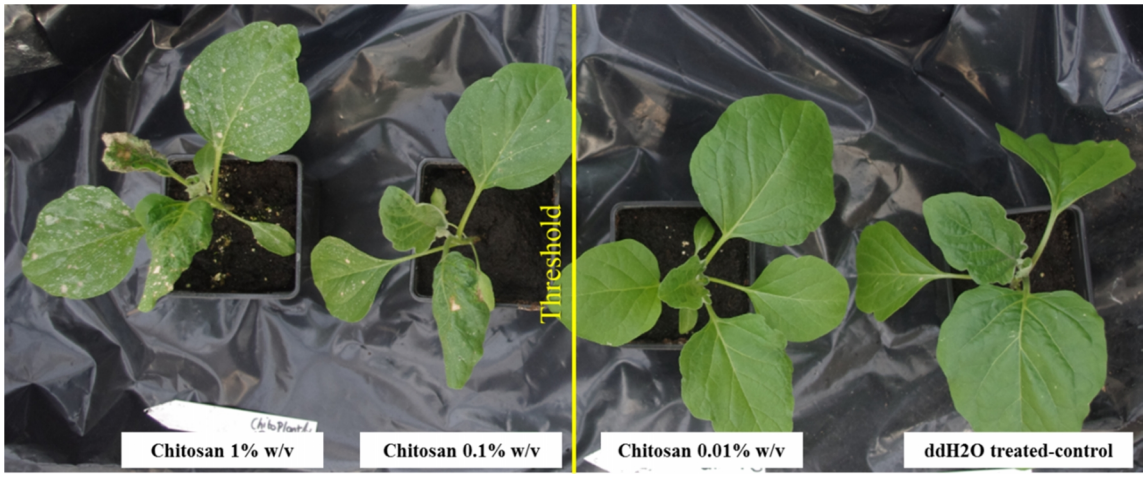
In order to achieve a more efficient defence strategy and less costly in terms of plant fitness, it is important, when using elicitors such as chitosan, to assess the effect of the concentration not only on the activation of plant endogenous defences, but also on the stress tolerance of the plant.

In some of the previous experiments in this chapter, chitosan treatments have been used at relatively high concentrations (1% and 0.1 % w/v) which did not have any detrimental effect on tomato growth although I decided to evaluate the possible effects on cell death, as it has been previously noticed (Iriti & Faoro 2009). Cell death was observed on tomato cotyledons (brown dots) after Chitosan1 (ChitoPlant) application on

1-2-week-old tomato seedlings (Picture 2.2) when applied at high concentrations (1% w/v). Thus, in order to investigate further whether this ChitoPlant-induced cell death is concentration-dependent, 4-week-old aubergine cv. Black Beauty plants were foliar treated with water solution (Control); and ChitoPlant at 0.01%, 0.1% and 1% w/v. Four days after treatment, plants were visually assessed for cytotoxic effects on the leaves surface.

Picture 2.2. Cell death/phytotoxic response in the cotyledons of 2 -week-old tomato plantlets 1 week after chitosan 1% w/v treatment (commercial formulation, ChitoPlant).

The highest concentrations of chitosan (1% and 0.1 % w/v) showed cytotoxic effect on treated and systemic leaves (Picture 2.3). However, this effect decreased with the concentration and had no visual effects on the lowest concentration (0.01% w/v). Thus, it seems to be a threshold concentration able to switch cell-death (cytotoxicity) in aubergine plantlets (Picture 2.3) as well as tomato. Thus, lower concentrations of chitosan were used in subsequent experiments of this chitosan formulation on tomato and other plants.



Picture 2.3. Cell death/phytotoxic response in the leaves of 4 -week-old aubergine plantlets 4 days after (from left to right) chitosan 1%, 0.1%, 0.01% w/v and ddH_2O treatment (commercial formulation, ChitoPlant).

2.4 DISCUSSION

2.4.1 Tomato cultivars respond in a different manner to long-lasting elicitor-induced resistance against *Botrytis cinerea*

In this chapter, the ability to induce durable resistance in tomato cvs. Moneymaker and Motelle against the *B. cinerea* of candidate elicitors BABA, BTH, MeJA, MeJA + BTH combined and two formulations of chitosan was investigated. Tomato cv. Moneymaker is a model wild-type susceptible cultivar to pests and pathogens used in numerous experiments throughout the years (Audenaert et al. 2002; Jupe et al. 2013; Wu et al. 2015). Tomato cv. Motelle is a near-isogenic cultivar to cv. Monemaker that carries introgressed regions containing aphid and nematode *Mi-1.2* resistance gene (Rossi et al. 1998; Wu et al. 2015). Interestingly, differential interactions and hence resistance levels of tomato cultivars to pests and pathogens have been identified, including cv. Moneymaker susceptibility and cv. Motelle resitance to *Fusarium oxysporum* f. sp. *lycopersici* (Ouyang et al. 2014); aphids and nematodes (De Ilarduya et al. 2001; Rossi et al. 1998). Furthermore, differences in the cultivar response to elicitor treatment and therefore subsequent induced resistance has been previously shown (Luna et al. 2016; Aranega-Bou et al. 2014). Thus, both cultivars were used to investigate differential interactions or possible similarities for elicitor-induced long-lasting (17 days after treatment) resistance against *B. cinerea*.

In general, both cultivars were susceptible to *B. cinerea* as seen in the control infections, however BABA, BTH, MeJA, MeJA + BTH and chitosan were able to induce long-lasting resistance against *B. cinerea* and differences in their response to the infection and the elicitors were found (Figures 2.1, 2.2 and 2.3). Elicitor-induced resistance appeared to be cultivar-dependent, as Chitosan2 and MeJA-induced more effective resistance in cv. Moneymaker than cv. Motelle whilst BABA-induced more effective resistance in cv. Motelle (Figure 2.3) which correlates with published results (Bruce et al. 2016) where BABA-treated and *B. cinerea* infected cv. Motelle plants were significantly less diseased than cv. Moneymaker. Interestingly, one chitosan formulation (ChitoPlant) significantly protected both cultivars at a similar level against

B. cinerea disease (Figure 2.3), which suggests a potential extrapolation of chitosan-induced resistance to other cultivars of tomato.

2.4.2 Chitosan has short-duration induced resistance effects in various crops and *in vitro* against *Botrytis cinerea*

As seen previously, chitosan (ChitoPlant) was able to induce long-lasting (17 dat) resistance in two tomato genotypes against *B. cinerea* infection (Figure 2.2). As chitosan-induced resistance was not cultivar-dependent in tomato it was hypothesized that chitosan-induced resistance will be similarly effective in other plants. However, for the long-lasting-induced resistance experiments chitosan was used at high concentration (1% w/v) which can ultimately have phytotoxic effects on the plant.

Thus, chitosan ability to induce resistance in different crops and more specifically chitosan-induced priming properties were investigated. Chitosan was able to induce short-duration (4-5 dat) resistance in *S. lycopericum*, *S. melongena*, *A. thaliana* and *N. benthamiana* at a range of concentrations. However, in *A. thaliana* chitosan-induced resistance followed a typical concentration-dependent curve whereas in tomato it did not depend on its concentration, although it was noticed that the high-concentration chitosan had a cytotoxic effect on tomato cotyledons (Picture 2.2). Chitosan-induced cell-death has been previously seen (Iriti & Faoro 2009). In aubergine, high concentration chitosan also had a detrimental cytotoxic effect which ultimately facilitated *B. cinerea* infection. This suggests a possible threshold in chitosan-priming for resistance that depends on its concentration and when exceeded, it may overstress plant defences to the benefit of necrotrophic pathogens such as *B. cinerea*, which correlates with *B. cinerea* host defence manipulation ability (Angulo et al. 2014; El Oirdi et al. 2011; Smith et al. 2014). Although these data showed that chitosan might prime crops for resistance against *B. cinerea*, this priming effect was concentration-dependent.

Thus, to determine the mode of action of chitosan (ChitoPlant) and to confirm whether it acts solely as a plant elicitor (PAMP/MAMP) or whether it has antifungal effects on the pathogen and/or it works in a combinatorial manner, the direct antifungal effects of chitosan against *B. cinerea* infection were investigated. An *in vitro* assay showed that chitosan abolished fungal hypha growth and spore germination under the high

concentrations tested whilst at low concentration chitosan showed no direct fungicide effect. Priming has been related to a cost-effective mechanism of induced resistance, in which the priming agent enhances the capacity of the host to movilize defence responses against pathogen attack avoiding direct defence induction in pathogen absence (van Hulten et al. 2006). Thus, it was hypothesized that chitosan-induced priming might be dose-dependent. Moreover, dose-dependent priming has been previously shown with other elicitors/priming agents, were application of high-concentrated MeJA had a greater antifungal effect than low concentrations on *Fusarium oxysporum* f.sp. *lycopersici* spore germination and mycelial growth and high doses of MeJA had detrimental effects on tomato physiological processes and decreased tomato protection efficiency against pathogen attack (Król et al. 2015).

Therefore, this suggest a concentration-dependence of priming of some elicitors (chitosan in particular), proposing a concentration threshold in which chitosan might not directly affect fungal growth but still prime the plant own defence mechanisms to challenge pathogen attack with minimal or no net cost to the plant.

2.4.3 Chitosan induces callose deposition in a concentration-dependent manner

It is well-known that plants can display relatively early acting defences, such as reactive oxygen species (ROS) production, including superoxide (O_2-) and H_2O_2, which are generated following the recognition of a range of pathogens, and they function as a threshold trigger for the hypersensitive response (HR) (Mouekouba et al. 2014) or callose deposition, a plant beta-1,3-glucan polymer, which is rapidly synthesized and deposited just beneath the sites of attempted pathogen penetration, and has long been considered as an important factor for plant penetration resistance against invading pathogens (Oide et al. 2013). These plant defences may be crucial for stopping or slowing pathogen expansion, thus leaving the plant time to trigger its fine tuned, late and durable defences (e.g. hormone pathways). To elucidate whether BTH, MeJA, chitosan and BABA act through inducing relatively early acting defences, callose deposition after elicitor treatment was examined. Interestingly, chitosan (ChitoPlant) was able to induce callose in both tomato cultivars whereas MeJA induced callose more in cv. Moneymaker and BABA induced callose more in cv. Motelle. These results seem to confirm an earlier theory of plant cultivar-dependence of elicitor-induced resistance (Walters et al. 2005). Furthermore, chitosan (ChitoPlant)-induced callose deposition in

tomato and *A. thaliana* does not follow a classical dose-response curve and the duration of the response is affected by the concentration. However, this duration may be inversely related to chitosan concentration. Again, this supports further the hypothesis of a concentration-dependency of elicitor (chitosan)-priming of defence responses.

2.4.4 Chitosan and MeJA may be able to fine-tune peroxidase and H_2O_2 activity in tomato against *Botrytis cinerea*

Reactive oxygen species (ROS) are quickly accumulated after pathogen attack (Temme & Tudzynski 2009), which makes them crucial for the disease development. However, even though the role of ROS against necrotrophic pathogens is unclear overall, together with callose accumulation they seem to be critical in the early stages of defence against infection (Finiti et al. 2014). The results of the extracellular peroxidase (POD) activity showed that the ABA deficient tomato mutant line *sitiens* had a strong POD induction when challenged with *B. cinerea*, which confirms earlier discoveries on *sitiens* resistance to *B. cinerea* is based on timely fine-tuned H_2O_2 production (Asselbergh et al. 2007). In tomato cv. Moneymaker, chitosan and MeJA appeared to prime POD in comparison with control infected plants, however there was a trend of chitosan-induced POD at early stages of the infection, while MeJA induced tomato peroxidases at later stages against *B. cinerea*, which indicates a difference in MeJA and chitosan modes of action against *B. cinerea*.

Furthermore, MeJA and to a less extent chitosan, were able to contain plant H_2O_2 accumulation on tomato infected leaves at later stages of the infection, whereas H_2O_2 in non-treated (control) leaves was not restricted at the infection sites and spread throughout a bigger area of the leaves, suggesting a possible change in host defence expression in response to *B. cinerea* challenge as stated before (El Oirdi et al. 2011). However, in order to test this theory, further experimentation is required.

2.4.5 MeJA and elicitor effects on hormone-related defence gene expression in tomato

It is important to test whether jasmonic acid (JA) is really involved in induced resistance in the *B. cinerea*-tomato pathosystem as JA is well-known a key hormonal mechanism against necrotrophs. MeJA priming properties and their ability to interfere with plant hormone cross-talk has been well-studied in tomato, *A. thaliana* and other

plant systems after pathogen attack (Biswas et al. 2014; Koornneef et al. 2008; Pluskota et al. 2007; Wang et al. 2009). For this reason, the priming properties of MeJA were assessed.

Cross-talk between salicylic acid (SA) and jasmonic acid (JA) signalling pathways plays an important role in the regulation and fine tuning of plant induced defences that are activated after pathogen attack (Dong 2004; Pozo et al. 2004; Koornneef et al. 2008). Moreover, there is evidence that *B. cinerea* can manipulate antagonistic effects between immune signalling pathways in plants in order to promote disease development (El Oirdi et al. 2011) but these manipulations may depend on the plant species and pathogen strain. However, it is not simply the manipulation of SA/JA pathways by a necrotrophic pathogen since exogenous application of SA to tomato plants can be sufficient to inhibit the JA-induced expression of genes encoding proteinase inhibitors (PI I and PI II), suggesting that SA targets the JA pathway downstream of JA biosynthesis (Doares et al. 1995).

In the present study MeJA induction of JA-dependent defence genes was used to investigate whether it can interfere in SA/JA cross-talk and prime tomato to potentially stop pathogen hormone manipulation. To test whether *B. cinerea* BcR16 strain is able to manipulate the antagonistic cross-talk between JA-SA pathways through NPR1 (El Oirdi et al. 2011), NPR1 expression was tested (Figure 2.22), a key regulator gene of systemic acquired resistance (SAR) (Pozo et al. 2004). NPR1 expression did not significantly differ among treatments, although there was a putative effect of MeJA-infected plants were NPR1 was repressed early during the infection (8 hpi) but not in control-infected (Ctrl + Inf) plants, which suggests that MeJA might reduce *B.cinerea* host immune system manipulation (El Oirdi et al. 2011). However, further experiments need to be done to test this theory.

Besides, gene expression analysis of two tomato JA-defence genes, Leucine aminopeptidase (Lap) A, a gene involved the plant-defence response against mechanical wounding, insect infestation, and in response to pathogen infection (Pautot et al. 2001), and lipoxygenase (Lox) D, a gene involved in jasmonic acid defences and biosynthesis (Scranton et al. 2013), the main defence phytohormone against necrotrophic pathogens such as *B. cinerea*, were investigated. LapA and LoxD transcript levels showed that both genes were repressed by both of the infected treatments. However, the LoxD

transcript was repressed by the pathogen during early stages of the infection and MeJA was able to prime it at 48 hpi, which suggests that MeJA is able to reduce JA-defence gene repression by the pathogen. Finally, as expected, MeJA-treated tomato plants highly triggered JA-regulated proteinase inhibitor gene (Pin1) in comparison with other elicitors, which again confirms the ability of these hormone-derivative elicitor to induce resistance against *B. cinerea* infection.

So far, plant hormone jasmonic acid was revealed as a key hormone in elicitor-tomato-*B. cinerea* interaction, which agrees with previous studies of JA key importance against necrotrophs (Aubert et al. 2015; Glazebrook 2005; Kravchuk et al. 2011). This also correlates with the *Bacillus subtilis* WT strain NRS1473 and the *Bacillus spp*. GB03-induced resistance phenotype (pathogenicity) assays were both strains were able to significantly delay *B. cinerea* necrotic lesion expansion (Figure 2.5). Interestingly, *Bacillus amyloliquefaciens* and *Bacillus subtilis* are well-known PGPR capable of inducing JA-dependent ISR in multiple crops and other plants (Choudhary & Johri 2009).

2.4.6 Costs of elicitor-induced resistance in tomato development: BABA-induced growth repression and chitosan minimal/non plant fitness costs

Elicitor-induced resistance can lead to potential costs and trade-offs in the plant (Luna et al. 2016; Redman et al. 2001; van Hulten et al. 2006; Walters & Heil 2007). Nevertheless, few studies have examined it in detail in crop systems. A soil drench treatment of 1-week-old tomato seedlings with a high concentrations of BABA (10 mM) and JA (1 mM) abolished plant growth and had lethal effects (Luna et al. 2016). Here it was demonstrated that a BABA treatment on tomato cvs. Moneymaker and Motelle seedlings showed a strong growth repression on both cultivars, reducing 32% the relative growth rate (RGR) in cv. Moneymaker (MM) and significantly higher 45% in cv. Motelle (Table 2.2 and Figure 2.25b). This confirms again a higher affinity of cv. Motelle towards BABA.

Furthermore, it was shown that ChitoPlant in particular and MeJA to a less extent (Tables 2.1, 2.2), were able to significantly reduced *B. cinerea* infection in two tomato cultivars with minimal fitness costs in plant growth. These results verify a cultivar-dependence to elicitor-induced fitness costs. Interestingly, water-soluble chitosan

(ChitoPlant) had a similar effect on the RGR of both cultivars, which again confirms a potential extrapolation of chitosan-induced resistance to other cultivars of tomato and other crops.

2.5 CONCLUSION

This chapter studies reveal how elicitors can significantly protect different plants against the aggressive fungal necrotroph *B. cinerea*. However, elicitor-induced resistance seems to be cultivar-dependent, which suggests that their effectiveness might be reduced to certain plant and pathogens. This chapter has shown that, among all elicitors evaluated, only low MW and water-soluble chitosan (ChitoPlant) can protect various crops and model plants against *B. cinerea* and induce callose deposition in two tomato cultivars independently of the genotype (Figures 2.3, 2.14 and 2.15), with minimal costs in the plant growth. Furthermore, it seems that chitosan-induced resistance does not follow a typical dose-dependent curve instead it has a 'priming-type' of response as lower concentrations (0.01 and 0.001 % w/v) induce resistance and prime callose for a longer period and more than the medium-high concentrations without having a phytotoxic effect. Furthermore, low-concentrated chitosan (0.01 % w/v) can induce resistance in different plant systems against *B. cinerea*, which shows that *A. thaliana*, *S. melongena* and *N. benthamiana* perceive chitosan in a similar way to *S. lycopericum*, which supports the hypothesis of common or homologous pathways in these plants against *B. cinerea* infection. In contrast, chitosan-induced resistance was not effective against the *A. thaliana* oomycete pathogen *Hyaloperonospora arabidopsidis* (*Hpa*), which suggests that chitosan-induced resistance might depend on the plant-pathogen system. Finally, it was demonstrated that ChitoPlant had a dose-dependent fungicidal effect *in vitro* against *B. cinerea* hypha growth and spore germination, being the low-concentrated (0.01 % w/v) the only concentration that did not have a direct antifungal activity.

Moreover, previous studies have shown that chitosan can induced resistance against various pathogens (Martínez-Castellanos et al. 2009; Iriti & Faoro 2008; Anusuya & Sathiyabama 2014; Benhamou et al. 1994; Ahmad et al. 2011). Last, although the diverse mechanisms of action of chitosan have been studied, which include activation of antifungal proteins (Muñoz & Moret 2010), oxygen-species scavenging and antioxidant activities, as well as the octadecanoid pathway activation (El Hadrami et al. 2010)

experiments that specifically address the role of priming in the complex chitosan-plant interaction framework are still lacking. There is therefore considerable potential for its use in new disease management strategies in important commercial crops, such as tomato, aubergine, pepper and oil seed rape and opens the possibility to include chitosan in IPM strategies as a 'priming agent'. Thus, the next chapter aims to characterise the role and molecular function of low dose chitosan (0.01 % w/v) as a priming agent in tomato plants for a faster, stronger, fine-tuned resistance to *B. cinerea*.

3 Chapter 3. Molecular characterisation of Chitosan-priming tomato against *Botrytis cinerea*: Transcriptomic and Phytohormone analysis

3.1 INTRODUCTION

3.1.1 Chitosan as a model and natural protector molecule

Chitosan is a copolymer of D-glucosamine and N-acetyl-D- glucosamine (Younes et al. 2014) with unique polycationic properties (Liu et al. 2000) and deacetylated derivative of chitin, which is highly abundant in nature and it is naturally present in the exoskeletons of shellfish, insects and fungal cell-wall (Islam & Datta 2015). Chitosan has multiple beneficial properties, including antimicrobial activity (Romanazzi et al. 2013), biocompatibility and biodegradability (Anusuya & Sathiyabama 2014) and it is non-toxic and renewable (Qin et al. 2002), which makes it a good candidate for extensive applications in pharmacy, medicine, agriculture, food and textile industries, cosmetics, and wastewater treatment (Younes et al. 2014). The many published studies demonstrate the considerable interest in this compound in pharmaceutical and biomedical applications, such as tissue engineering, obesity treatment and immunology due to its unique properties (Cheung et al. 2015). In the last decade, chitosan has been extensively used to protect horticultural crops, cereals, ornamentals, fruit and medicinal crops against pathogen attack (Pichyangkura & Chadchawan 2015; El Hadrami et al. 2010; Anusuya & Sathiyabama 2014; Romanazzi et al. 2013). In crop protection, chitosan synergistic effects have also been shown, such as its synergy with *Cryptococcus laurentii* on inhibition of *Penicillium expansum* infections in apple fruit (Yu et al. 2007). Chitosan was approved by the European Commission as a "basic substance" in 2014 making it a very useful candidate for crop protection (http://ec.europa.eu/food/plant/pesticides/eu-pesticides-database).

The biological activity of chitosan depends on the host, its degree of deacetylation, its concentration and structure (Iriti & Faoro 2009; Limpanavech et al. 2008; Lin et al. 2005), and the chemical composition of the substrates (El Hadrami et al. 2010). However, one of the main disadvantages that limits chitosan application is its low

solubility in neutral and alkaline solutions (Cheung et al. 2015). Chitosan antimicrobial properties can be affected by its poor solubility in non-acidic medium (Liu et al. 2000; Badawy & Rabea 2014) but different molecular weights chitosan derivatives have been developed with higher water solubility that exhibit antimicrobial activities (Cheung et al. 2015; El Hadrami et al. 2010; Liu et al. 2000). Chitosan physicochemical complexity can ultimately affect plant recognition, which makes it a complex system for plant application and absorbance. Previous experiments infiltrated chitosan into the plant to ensure recognition and/or an effect (Ahmad et al. 2011; Scalschi et al. 2015) and others needed complex ways to dissolve chitosan oligosaccharides before plant application by dissolving practical grade chitosan in acetic, glutamic, formic and hydrochloric acids (Romanazzi et al. 2013).

As described in Chapter 2, for transcriptomic analysis chitosan application has been carried out by directly dissolving water-soluble chitosan (ChitoPlant, commercial formulation) into distilled water and with the help of a surfactant (e.g. Silwet L-70, Tween20) foliar spraying directly onto the plant, whereas other chitosan polymers need to be dissolved into acidic-soluble solutions.

In order to efficiently fight against potential pathogenic microbes, plants have evolved to sensitively and rapidly recognize would-be pathogens through cell surface-localized receptors, termed pattern-recognition receptors (PRRs) (Zipfel 2014). Chitosan can be recognized through plant PRRs, including a chitosan-binding protein and putatively the chitin elicitor-binding protein (CEBiP) (Iriti & Faoro 2009), and act as a pathogen/microbe-associated molecular pattern (PAMP/MAMP), being able to activate broad spectrum, long-lasting and systemic defence mechanisms (Iriti & Faoro 2008). It can also mediate basal resistance in the plant called PAMP-triggered immunity (El Oirdi et al. 2011).

Chitosan can behave as a general elicitor, directly inducing non-host resistance or through priming the plant for systemic acquired resistance (SAR) (Iriti & Faoro 2009). Studies have shown the diverse mechanisms of action of chitosan, which include activation of antifungal proteins (Muñoz & Moret 2010), oxygen-species scavenging and antioxidant activities (El Hadrami et al. 2010). There are studies that recognize the ability of chitosan of inducing H_2O_2 followed by a concentration-dependent necrotic cell death (Iriti & Faoro 2009). Chitosan defence-eliciting capacities in plants include the expression of unique early responsive and defence-related genes, as part of the PAMP-

triggered immunity (PTI), which include the accumulation of cytosolic H+ and Ca2+, activation of MAP-kinase (MAPK) signalling cascades, oxidative burst, synthesis of abscisic acid (ABA), jasmonic acid (JA), phytoalexins and pathogenesis-related (PR) proteins and the ability to induce callose formation (El Hadrami et al. 2010; Iriti & Faoro 2009; Romanazzi et al. 2013). Furthermore, despite plant-necrotrophic pathogen interactions being poorly understood, recent studies have demonstrated the importance of PRR-mediated PAMP recognition and PTI in resistance against necrotrophic fungi (Wang et al. 2014).

Experiments that specifically address the role of priming in the complex chitosan-plant interaction framework are still lacking. Chapter 2 experiments showed water-soluble chitosan priming properties, where induced resistance phenotypic (pathogenicity assays) demonstrated that among all elicitors analysed, water-soluble chitosan (ChitoPlant) was able to induce long-lasting resistance and callose deposition in tomato cvs. Moneymaker and Motelle and short-duration resistance in other Solanaceae such as *S. melongena* and *N. benthamiana* and the model plant *A. thaliana* against *B. cinerea*. Furthermore, ChitoPlant-induced resistance did not have a negative effect on the growth of these tomato cultivars, nor did it depend on the tomato cultivar as it was shown that ChitoPlant significantly decreases *B. cinerea* lesion expansion similarly in both cultivars. For this reason, in this chapter the aim was to investigate 'chitosan-primed and triggered' (Hilker et al. 2016; Luna et al. 2012) tomato response against *B. cinerea* through a large-scale double transcriptomic analysis and small-scale HPLC/MS phytohormone assay.

3.1.2 Tomato-*Botrytis cinerea* interaction

Tomato is an economically important crop that is susceptible to many pest and pathogens, such as fungi, oomycetes, bacteria, viruses, nematodes and insects (Chen et al. 2013; Jupe et al. 2013; Pautot et al. 2001; Scranton et al. 2013). During the last decade, tomato has become one of the most important model crops to study plant-pathogen, including tomato-*B. cinerea*, interactions (Arie et al. 2007). As explained in Chapter 1, many research studies have been conducted to investigate tomato immune response against *B. cinerea*. Some of these studies include experiments with wild resistant tomato relatives, such as *Solanum lycopersicoides* and *Solanum habrochaites* (Finkers et al. 2007; Guimarães et al. 2004; Smith et al. 2014; Chen et al. 2013), large

scale transcriptomic and metabolomic analysis of tomato infected with *B. cinerea* (Blanco-Ulate et al. 2013; Chen et al. 2013; Smith et al. 2014; Guimarães et al. 2004; Asselbergh et al. 2007; Diaz et al. 2002; Audenaert et al. 2002) and single gene-based reverse genetics studies (Li et al. 2014; Liu et al. 2014; Li et al. 2015; Li et al. 2015; Liu et al. 2014).

However, few studies have investigated elicitor/priming agents mode of action in tomato-induced resistance against *B. cinerea* through large-scale transcriptomic analysis (Finiti et al. 2014) and to date full understanding of tomato defence mechanisms against this pathogen is lacking (Asselbergh & Höfte 2007; Diaz et al. 2002). *B. cinerea*, it is considered to be the model necrotrophic pathogen but its infection strategy is not fully understood. New insights about the necrotroph complex infection strategy have been unveiled through –omics analysis, including *B. cinerea* early extracellular proteome, termed secretome, where novel virulence factors (unspecific genes involved in pathogenicity such as germination, cell-wall penetration, necrosis and disease promotion) where found involved in germination of fungal conidia, including mannitol-1-phosphate dehydrogenase, 6,7-dimethyl- 8-ribityllumazine synthase and uracil phosphoribosyltransferase (Gonzalez-Rodriguez et al. 2014). This demonstrates the importance *B. cinerea* early genome machinery and that the effective establishment and the outcome of infection might be dependent of the initial stages of the infection (Espino et al. 2010). Moreover, previous studies have also revealed *B. cinerea* virulence factors involved in necrosis and cell-wall degradation, such as xylanase Xyn11A; endopolygalacturonases (endoPGs), glucoamylase BcGs1 (Zhang et al. 2015; Leone 1992; Brito et al. 2006). Furthermore, some of these virulence factors are considered to behave as effector molecules, such as BcNEP1,2-like proteins and, recently revealed, smallRNAs (BcsRNAs) that *B. cinerea* uses to induce cell death and suppress host immunity respectively (Schouten et al. 2008; Weiberg et al. 2013). All this demonstrates the *B. cinerea* complex infection strategy where one third of its secretome is still unknown (Fillinger & Elad 2015). Therefore, this chapter's main aim is to investigate downstream molecular mechanisms by which chitosan primes for resistance to *B. cinerea* in tomato through a novel approach large-scale double transcriptomic analysis that will study both the host and the pathogen transcriptomic response to chitosan simultaneously.

3.2 MATERIALS AND METHODS

3.2.1 *Botrytis cinerea* infection progress in *Solanum lycopersicum*

Time series was performed to help select key asymptomatic time points after infection to evaluate *B. cinerea* infection progress in tomato plants. Whole detached leaves (1-2) of 4-week-old untreated tomato cv. Moneymaker plants were infected with a spore solution of *B. cinerea* (2 x 10^4 spores/ mL) as described previously (see Chapter 2 Pathogenicity assay (Worrall et al. 2012)). Leaf discs were sampled at 1, 3, 6, 9, 12 and 24 hpi and subsequently fixed in 96 % ethanol and left overnight prior to do stain. One day later, double staining (aniline blue + calcofluor) was performed. Briefly, leaf discs were soaked in 0.05% w/v aniline solution and 0.001% v/v calcofluor for approximately 15 minutes, staining solution was replaced with fresh 0.005 % aniline (without calcofluor) and incubated at room temperature overnight in the dark. Slides were prepared in fresh aniline solution and viewed under 365 nm excitation light with DM 400 LP.

3.2.2 Large-scale transcriptome (microarray) analysis of chitosan-treated and infected *Solanum lycopersicum* plants with *Botrytis cinerea*

Tomato cv. Moneymaker seeds were placed into propagators containing Bulrush soil (non-insecticide regular mix, see Chapter 2 M&Ms) and a layer of vermiculite on the top and left at 20 °C for 1-2 weeks until germination. Germinated seeds were transplanted to individual pots containing pesticide-free compost and grown in growth cabinets under controlled tomato standard growth conditions (16h- 8h/ day- night cycle; 23°C/ 20°C).

Four-week-old tomato cv. Moneymaker plants were treated with ddH_2O solution and 0.01% w/v of ChitoPlant (termed ‘chitosan’ from now onwards) in 0.01% Tween20, 4 days prior fungal infection, by spraying the solution onto the plants. Four days after treatment, 1-2 whole leaves per plant were excised and subsequently infected with a spore solution of *B. cinerea* (2 x 10^4 spores/ mL) or ddH_2O for the mock, by drop inoculation (Figure 3.1).

To investigate the chitosan-induced resistance phenotype before transcriptome analysis, 1-2 whole detached leaves of 6 extra chitosan-treated + *B. cinerea*-infected (Chito+Inf) plants and 6 extra ddH_2O-treated and *B. cinerea*-infected (Inf) tomato plants were kept for 3 days in constant darkness and high humidity (80-90%) at 21 °C and subsequently assessed for the resistance phenotype. Necrotic lesions of the infected leaves were measured at 2 and 3 dpi with an electronic ruler to quantify the symptomatic differences between control and chitosan-treated plants.

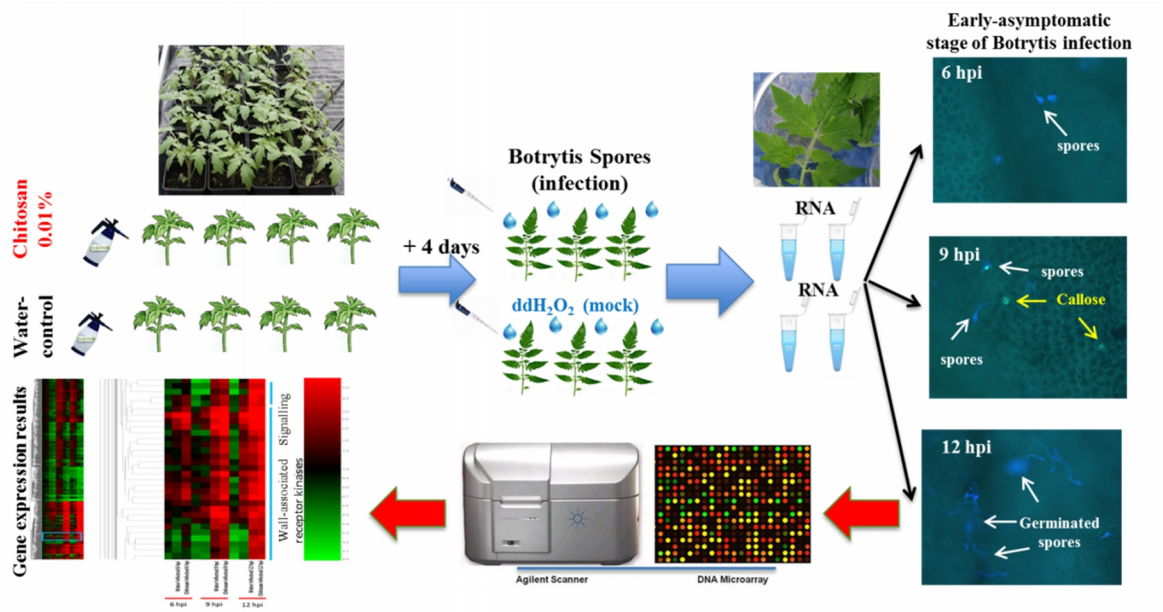


Figure 3.1 Experimental design of the transcriptomic (microarray) analysis of chitosan-primed and *B. cinerea*-inoculated tomato plants. Four conditions (3 + control) were analysed: **(i)** ddH_2O-treated and non-infected control plants (Control); **(ii)** ddH_2O-treated and *B. cinerea*-infected plants (Water+Inf or Inf); **(iii)** chitosan-treated and non-infected/mock plants (Chito+Mock) and; **(iv)** chitosan-treated and *B. cinerea*-infected plants (Chito+Inf). Four days after treatment (dat) leaf discs from four plants per treatment were sampled at three early (asymptomatic) time points, at 6, 9 and 12 hours post-inoculation (hpi) of *B. cinerea* spores or mock water-control.

Leaf discs from four plants (4 biological replicates) per treatment were sampled for total RNA extraction at three early (asymptomatic) *B. cinerea* infection stages (6, 9 and 12 hpi). Total RNA was extracted with RNeasy Plant MiniKit (Qiagen) using recommended protocols. Spectrophotometry (Nanodrop) was used to estimate concentration and determine if contaminants were present by analysing 260/230 nm and 260/280 nm ratios. RNA integrity was tested using a Bioanalyzer 2100 (Agilent), using

the manufacturer's recommended protocol with RNA 6000 Nano chips. Once concentration and RNA Integrity Number (RIN) were determined, total RNA was hybridized to microarrays, processed by the Genome Technology (GT) group of The James Hutton Institute.

All high-throughput transcriptome analysis utilised a joint Agilent 60k array design made with 16,365 *B. cinerea* and 34,616 *S. lycopersicum* oligonucleotide probes. Sets of 60-mer probes were designed by the GT group to represent the entire transcriptome complement of both species using default parameters in Agilent eArray software (https://earray.chem.agilent.com/earray/) and formatted in 8x 60k slides (Agilent design ID 074103). Microarray processing and data extraction was performed by the GT group using recommended protocols. Briefly, two-channel microarray processing was utilised, labelling total RNA with either Cy3 or Cy5 fluorescent dyes (Low Input Quick Amp Labelling Protocol v. 6.5, Agilent). Following purification of labelled cRNA, levels of dye incorporation were determined by spectrophotometry (Nanodrop). Microarrays were hybridised overnight as recommended, according to the experimental plan. After washing, microarrays were scanned using an Agilent G2505B scanner at 5 micron resolution to capture images from both dye signals. Images were subsequently imported into Feature Extraction software (v. 10.7.3.1; Agilent), and data extracted using default parameters for 2-colour images. Data were QC checked prior to importing into Genespring software (v. 7.3; Agilent) for subsequent pre-processing and statistical analysis. Data for the host and pathogen were imported into separate instances of Genespring to ensure correct normalisation and data filtering procedures could be applied. In addition, all data were re-imported into Genespring following initial Lowess global normalisation of each data type, and subsequently processed as single-colour data to reduce statistical restrictions to analysis.

3.2.3 *Solanum lycopersicum* microarray data analysis

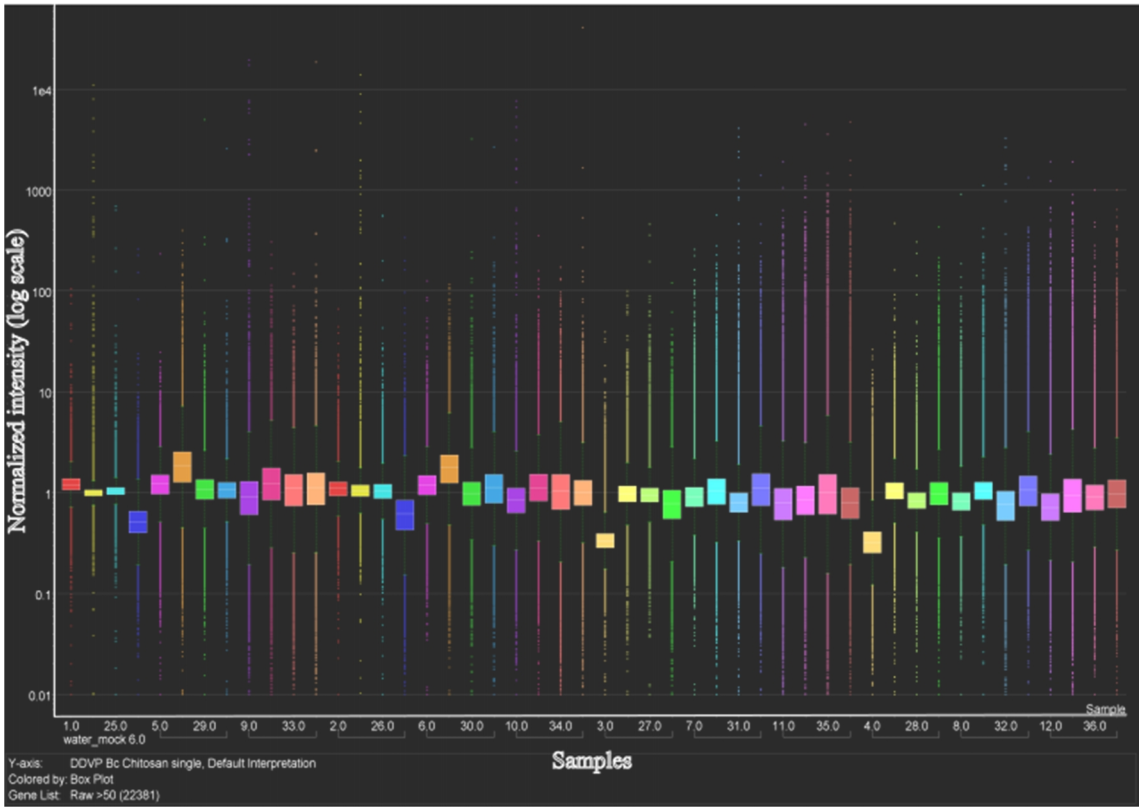
Data were pre-processed for quality control and to filter out those probes which did not have consistent signal for any sample in the experiment before being loaded into Genespring (version 7.3; Agilent Technologies) software for analysis. Similarity of replicate microarrays was visualised using box-whisker plots (Figure 3.2).

Data was filtered to include only probe sets for which three out of four probe signals from replicate arrays exceeded the set arbitrary minimum signal (Raw > 50). This filter narrowed down the set of reliable probes to 22,381 transcripts from the original 34,616.

A statistical test was used to identify a global list of differentially-expressed genes (DEGs), which was subsequently used for specific pairwise analyses. This was performed by 2-way Analysis of Variance (ANOVA), using the experimental factors 'Treatment' (3,713 DEGs) and 'Time' (6,920 DEGs), also generating the 'Treatment-Time interaction' (186 DEGs) (Figure 3.2b). A cut-off P-value ≤ 0.01 was used, along with Benjamini-Hochberg false discovery rate correction to adjust for the large numbers of tests. The 2-way ANOVA used identifies genes changing with respect to 'treatment', 'time' and the 'interaction between treatment and time'; hence helping to identify genes with a shift in their expression on treatment between each time point and another.

The 2-way ANOVA identified a global set of 8,471 differentially-expressed genes/probes (DEGs), with some overlap between gene lists (Figure 3.2b). Subsequently, pairwise Student's T-test comparisons were performed (Volcano plots: P-value ≤0.05, 2-fold cut-off) on the DEGs for the three test treatments (Chito+Mock, Inf and Chito+Inf) compared to control treatment (Water-treated+non-infected/mock) at each time point. Lastly, to identify differentially expressed genes responding uniquely to (i) chitosan without infection, (ii) infection only, and (iii) chitosan+ infection, Venn diagrams were utilised at each time point.

(a)



(b)

2-way ANOVA (p-value <=0.01; B&H correction)	DEGs
Total (ANOVA all)	8471
Treatment	3713
Time	6920
Interaction	186

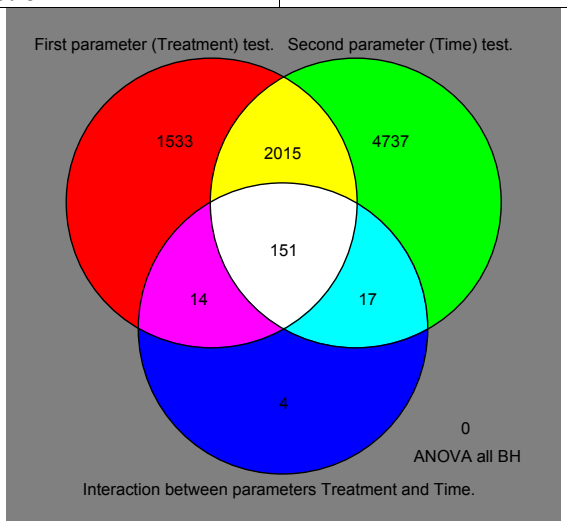


Figure 3.2 Agilent Microarray statistical analysis performed with GeneSpring (a) Box-whisker plots. Similarity of replicate microarrays was visualised using GeneSpring software. Data was normally distributed. Shifts in boxes in plots indicate limitations of the normalisation process, acceptable for Agilent arrays; (b) 2-way combined Analysis of variance (ANOVA) for 'treatment', 'time' and

'treatment-time interaction' factors. The 2-way ANOVA helped to identify 8,471 differentially-expressed genes/probes

3.2.3.1 MapMan custom diagrams and Panther GO term enrichment analysis

All statistical analysis to identify DEGs were performed with GeneSpring software (v. 7.3; Agilent) as explained above. MapMan software (Thimm et al. 2004) was used to visualise DEG products in the context of biological pathways, using default settings. Chito+Inf and Inf specific DEGs lists generated using Venn diagrams were imported into MapMan. Overview pathways were selected (*S. lycopersicum* ITAG2.3 gene annotation release) and custom diagrams were generated to visualize the selected data and show the differentially up/down-regulation of pathways of Infected vs. Chitosan+Infected treatments.

Functional enrichment analysis was performed with Panther software (Thomas 2003) by plotting Chito+Inf and Inf specific DEG lists at 6, 9 and 12 hpi generated with GeneSpring. Biological processes and molecular functions PANTHER Overrepresentation Test (release 20170413) was selected against *Solanum lycopersicum* (all genes in database); and Bonferroni correction for multiple testing was used.

3.2.3.2 Microarray validation (RT-qPCR)

Validation of *S. lycopersicum* transcriptomic analysis was performed by RT-qPCR of 9 candidate diferentially expressed genes (DEGs) included in the ANOVA and subsequently compared with gene expression values from the Microarray. Selected RNA samples (same from the Microarray) were Dnased with TurboDnase (ThermoFisher) and complementary DNA (cDNA) was synthesized from 2.5 µg of total RNA using Superscript III reverse transcriptase (Invitrogen) and random hexamer/Oligo DT primers.

qRT-PCR reactions were performed with specific *S. lycopersicum* primers (Sigma-Aldrich) of *SlACRE75*, *SlACRE180*, Avr9/Cf-9 rapidly elicited gene 75/180; *SlPOD5*, Peroxidase 5; *SlCHI2*, chitinase2; *SlLBD42*, lateral organ boundaries domain protein 42, *SlPtoSer/threK*, Pto-like, Serine/threonine kinase protein, resistance protein; and *SlGST*, Glutathione S-transferase at 9 hpi; *SlRLKase*, Receptor serine/threonine kinase-like protein (at 12 hpi), against two reference genes (*SlActin-like* and *SlUbiquitin*).

Gene primers and probes were designed using Universal Probe Library (UPL) assay design center (Roche Diagnostics Ltd.) and qRT-PCR was performed using FastStart Universal Probe Master Mix (Roche). Amplification and detection of specific products were performed according to the manufacturer instructions, with the following cycle profile for the UPL primers: denaturation step at 95° C for 10 min; 40 cycles of 95° C for 15 sec, annealing and extension at 60° C for 1 min. Each qPCR reaction contained at least two non-template controls. All reactions were run in technical triplicates for each biological replicate and the mean values were used for quantification. The relative quantification of target genes was determined using the Pfaffl method (Pfaffl 2001).

3.2.4 *Botrytis cinerea* microarray data analysis

As previously stated this transcriptomics analysis utilised a joint Agilent 60k array design made with 16,365 *B.cinerea* probes and 34,616 *S. lycopersicum* probes. For the analysis of the pathogen, a threshold signal of more than 50 was applied (Raw >50) for 3 out of 24 samples, leaving 2,015 probes passing the cut-off (of the 16,365 total *B. cinerea* probes). Two variables were taken into account, time and treatment (chitosan or water), for 2-way ANOVA (P-value $\leq$ 0.05, Benjamini-Hochberg adjustment). This restricted the list of genes to just 18, which was deemed too restrictive, therefore simple pairwise t-tests were performed, as described below.

Due to early stages of the infection and possibly low amount of fungal biomass, the level of pathogen gene expression was low; therefore, for this reason it was decided to work with the Raw > 50 probe list.

Pairwise statistical tests (Volcano plots: P-value $\leq$0.05, 2-fold cut-off; parametric test) were performed, comparing water-treated + infected (Inf) with chitosan-treated + infected (Chito+Inf) samples at each time point. No multiple testing correction was performed for the pairwise t-tests as this again was too restrictive, with no genes coming through as significant with Benjamini & Hochberg multiple testing correction. Therefore, the implications will be that there are potentially a higher number of false positive differentially expressed genes (DEGs) for *B. cinerea* transcriptomic analysis; hence the lists have to be considered as preliminary and with caution. However, the host and not *B. cinerea* transcriptome was the main focus of this study as stated in Results.

3.2.5 Phythormone analysis: HPLC/MS/MS

3.2.5.1 Elicitor treatments and pathogenicity assay

Tomato cv. Moneymaker seeds were grown under standard conditions as explained in Chapter 2 (2.2.3). Four-week-old tomato cv. Moneymaker plants were treated, 4 days prior fungal infection, with ddH_2O solution, 0.01% w/v of chitosan (in 0.01% Tween20), MeJA (0.1 mM) (in 0.01% Tween20) and a combination of chitosan 0.01% + MeJA (0.1 mM) (in 0.01% Tween20) by spraying the solution onto the plants. Four days after treatment, 1-2 whole leaves per plant were excised and subsequently infected with a spore solution of *Botrytis cinerea* (BcR16 strain) by droplet infection (2 x 10^4 spores/ mL) and/or water inoculation as a control (mock).

Previous HPLC/MS analysis, 1-2 whole detached leaves of 6 extra plants of Chito+Inf (chitosan-treated + infected plants), MeJa+Inf (MeJA-treated + infected plants) and Inf (ddH_2O-treated + infected tomato plants) were kept 3 days at constant darkness, high humidity (80-90%) and 21 °C and subsequently assessed for resistance phenotype. Necrotic lesions of the infected leaves were measured at 2 and 3 dpi with an electronic ruler for significant differences between Inf and Chito+Inf (chitosan-primed) tomato plants (Figure 3.24).

3.2.5.2 HPLC/MS analysis

For the HPLC/MS analysis, tomato leaf tissue was harvested at 6, 9 and 24 hours after infection/inoculation and subsequently frozen in liquid nitrogen. Tissue was then stored at -80°C then freeze dried. Freeze dried samples were ground (adding a tungsten ball) in a beat beater. Ten mg of each sample was used for hormone extraction. Hormones (JA, SA and ABA) were extracted following a standard protocol (Forcat et al. 2008). Briefly, 10 (+/- 0.1) mg freeze dried leaf powder was accurately weighed and extracted in 2 x 0.4 mL 10% methanol containing 1% acetic acid. Two extraction stages were performed to ensure effective extraction of the hormones. For accurate quantification, solvent A also contained the following amounts of stable isotope labelled hormone standards: 20 ng 2H6 salicylic acid (SA) (C/D/N Isotopes, Quebec, Canada), 10ng 2H5 jasmonic acid (JA) (C/D/N Isotopes, Quebec, Canada) and 10 ng 2H6 abscisic acid (ABA) (OlChemlm, Czech Republic). Samples were vortexed every 10 minutes, for 30 minutes. Each step involved incubation on ice followed by 15 minutes sonication in an ice water bath. Supernatants from both extractions were pooled after centrifugation (10

min at 16,100 x g, 4 °C) followed by filtering through a 0.4 µm (RC) syringe filter (Phenomenex, UK). Finally, quantitative analysis of plant hormones was performed using an Agilent 6420B triple quadrupole (QQQ) mass spectrometer (Technologies, Palo Alto, USA) hyphenated to a 1200 series Rapid Resolution HPLC system. Five µL of sample extracts were loaded onto an Eclipse Plus C18 3.5 µm, 2.1 x 150 mm reverse phase analytical column (Agilent Technologies, Palo Alto, USA).

3.2.6 Mass-spectrophotometer data analysis

Data analysis was undertaken using Agilent Mass Hunter Quantitative analysis software for QQQ (Version B.07.01). Accurate quantification of ABA, SA and JA used the deuterated internal standards added during sample extraction (Forcat et al. 2008). For the other compounds (e.g. jasmonic acid-isoleucine (JA-Ile)) normalised peak areas were compared and concentrations were calculated using standard concentrations curves.

3.2.7 Chitosan-IR pathways in *Arabidopsis thaliana* against *Botrytis cinerea*

With the collaboration of Dr. Estrella Luna (Sheffield University), *A. thaliana* wild type (WT) Columbia-0 and *Agrobacterium tumefaciens* transfer-DNA (T-DNA) insertion *A. thaliana* lines disrupted/mutated (knock out (KO)) in *npr1* (Thomma et al. 1998) (SA KO line), *npr1-pmr4* (Nishimura et al. 2003) (SA and callose KO line), *jar1* (Aranega-Bou et al. 2014; Staswick et al. 2002) (JA KO line) and *rbohD/F* (Torres et al. 2002) (ROS KO line) genes were mass-seeded on soil (Sheffield compost, see M&M Chapter 2), grown in a controlled environment cabinet and cultivated under Arabidopsis standard growth conditions (8h-day (21ºC) and 16h-night (18ºC) cycle at ~60% relative humidity (RH).

Ten-day-old plants were transplanted to another pot with a total of 5 plants per pot. In order to determine which hormone pathways are involved in chitosan-IR in *A. thaliana* against *B. cinerea*; 5-week-old Arabidopsis WT and KO plants were treated, 4 days prior fungal infection, with chitosan 0.01% w/v and ddH_2O (in 0.01% Silwet L-77 as an adjuvant) by spraying the solution onto the plants. Four days after treatment, plants were infected with *B. cinerea* by drop inoculation of a solution containing 5 x 10^4 spores/ mL in ½ strength PDB. Disease was scored at 2 and 3 dpi by measuring the

diameter of the symptoms (necrotic lesions) with an electronic ruler. Representative pictures were taken at 2 and 3 dpi.

3.3 RESULTS

3.3.1 Identification and characterisation of chitosan-induced priming for resistance against *Botrytis cinerea* in *Solanum lycopersicum*

To accomplish a global overview of the gene expression response of chitosan-primed and *B. cinerea* infected tomatoes, a whole transcriptomic approach provides an ideal tool to identify multiple expected and novel pathways that will be affected by the priming agent and also by the pathogen. This can be achieved using a Microarray chip analysis where specific pathways can be further investigated e.g. specific defence-related hormone analysis by proteomics or metabolomics such as HPLC/MS, which will add value towards the transcriptomic data.

Therefore, a double (host and pathogen) large-scale transcriptome study was designed to characterise the transcriptomic response of chitosan-primed tomatoes to *B. cinerea* infection and to investigate chitosan low-concentrated (0.01% w/v) mode of action, with potential priming properties identified in Chapter 2, and its role in priming tomato against the fungal aggressive necrotroph *B. cinerea*. The aim was to unveil the defence gene expression pattern of chitosan-primed and *B. cinerea*-inoculated tomato plants, and in doing so, identify potential specific pathways and genes that might be involved in the chitosan-induced resistance phenotype.

3.3.1.1 *Botrytis cinerea* infection progress on *Solanum lycopersicum*

A standardised infection experiment was used to confirm fungal infection progress in 4-week-old tomato cv. Moneymaker detached leaves and to identify most appropriate asymptomatic stages during *B. cinerea* infection, in which leaf discs were double stained with aniline blue + calcofluor and observed under UV excitation.

In *B. cinerea*-infected leaf tissue, germinating spores can be observed as early as at 3 hpi; bright callose deposits were first present at 6 hpi in tomato epidermal cells, suggesting plant recognition and defence at this early time point (Figure 3.3). At 9 hpi germinated spores penetrated the tomato leaf surface whilst there was a higher hypha growth at 12 hpi. However, no callose deposits were observed at 9 and 12 hpi. At 24 hpi

hyphae covered tomato epidermal cells and fungal penetration sites can be seen on the epidermis (Figure 3.3).

In order to achieve a wide spectrum of "primed genes" during infection and therefore to cover a gene expression profile that encompassed the 'primed and triggered state' (Mauch-mani et al. 2017; Hilker et al. 2016) of the plant during the infection, 6, 9 and 12 hpi were chosen as the more suitable asymptomatic early time points for the global gene expression analysis via microarray technology.

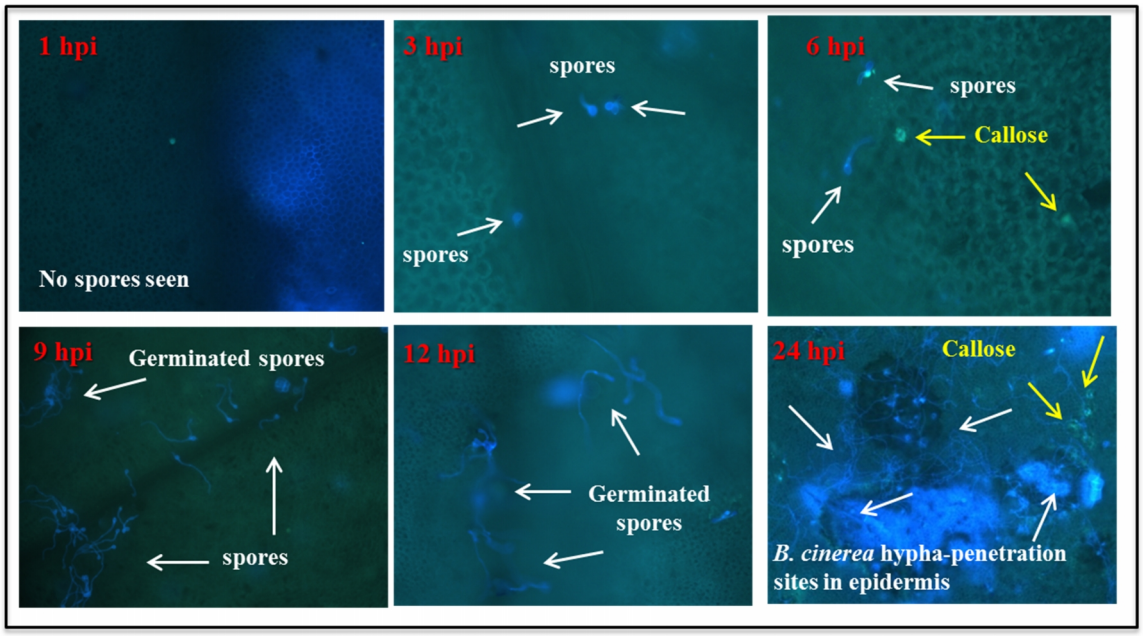


Figure 3.3 *B. cinerea* infection progress on tomato infected leaves at 1, 3, 6, 9, 12 and 24 hours post-infection (hpi) with *B. cinerea* spores inoculum (2 x 10^4 spores/ mL). Whole leaves (leaf discs) were harvested for double staining (aniline blue + calcofluor) and then pictures were taken under fluorescence microscopy at 4x and 10x magnifications.

3.3.1.2 Chitosan-induced resistance phenotype

Prior to whole transcriptome analysis, a chitosan-induced resistance phenotype was assessed from 1-2 whole detached leaves of chitosan- or ddH_2O-treated tomato plants (six of each), challenged with *B. cinerea* spore inoculum. Necrotic lesions of the infected leaves were measured at 2 and 3 dpi. Application of chitosan (Chito+Inf) significantly decreased lesion size in comparison to water-treated and infected (Inf) tomato plants at 2 and 3 dpi (Figure 3.4), thereby demonstrating the resistance phenotype was evident after 2 days.

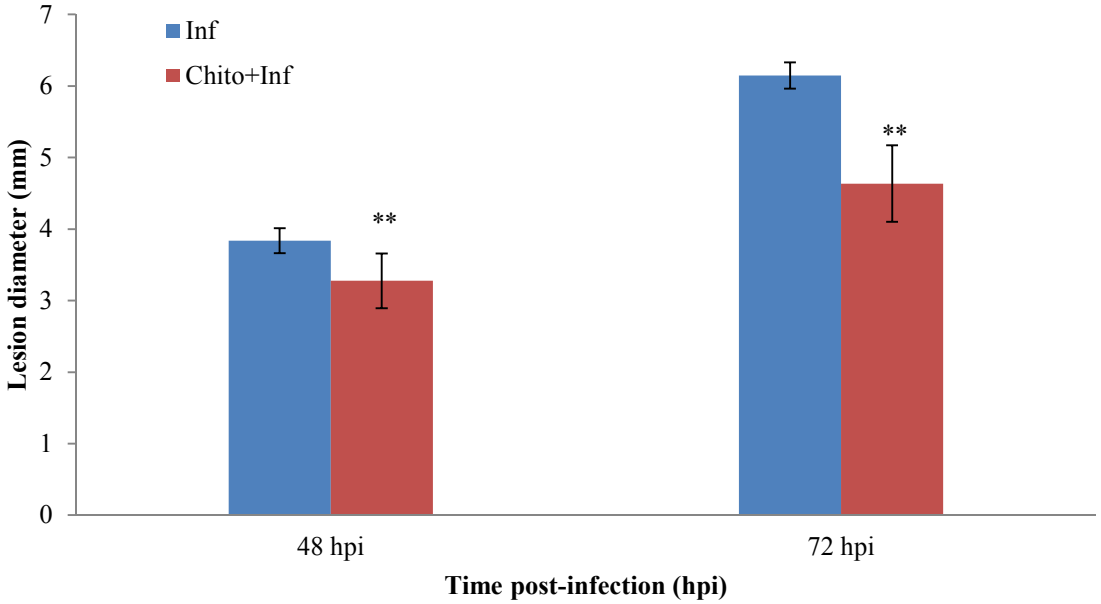


Figure 3.4 Quantification of chitosan (ChitoPlant)-induced resistance against *Botrytis cinerea* (2 x 10^4 spores/ mL) at 2 and 3 dpi in tomato cv. Moneymaker. Values presented are means ± SEM. Asterisks indicate statistically significant differences between both treatments (Inf and Chito+Inf) (Student's T-test $P<0.001$ at 2 and 3 dpi, $\alpha=0.05$).

3.3.2 Transcriptomic analysis of chitosan-primed and *Botrytis cinerea*-infected *Solanum lycopersicum* plants

3.3.2.1 Identification of differentially expressed genes (DEGs) primed by chitosan against *Botrytis cinerea* in *Solanum lycopersicum*

The main purpose of this study was to identify molecular pathways and specific genes whose expression in response to *B. cinerea* infection is altered by a putative priming agent (chitosan). This will ultimately help to decipher pathways involved in chitosan-priming for resistance tomato plants against *B. cinerea*. Following the application of the appropriate filtering and statistical analysis (ANOVA), among the 34,616 *S. lycopersicum* probes spotted on the microarray, a total of 8,471 differentially-expressed genes (DEGs) were identified among all three treatments and time points. This global list of DEGs was subsequently used for focussed pairwise analysis to identify probes changing between treatments at each timepoint. Volcano Plots (pairwise Student's T-test, in combination with fold-change) were used for every condition compared to ddH_2O-treated and non-infected/mock inoculated control (**Control**). Thus, the three treatments studied were: (i) **Inf** (ddH_2O-treated and *B. cinerea* infected plants); (ii) **Chito+Mock** (chitosan-treated and non-infected/mock inoculated plants) and; (iii)

Chito+Inf (chitosan-treated and *B. cinerea* infected plants), as explained in M&M. To identify genes whose expression is differentially regulated by *B. cinerea* infection and altered by chitosan, Venn diagrams were used to classify which DEGs are unique to each of the conditions per time point (Figure 3.5).

Chitosan had a strong impact in tomato gene expression; the combination of chitosan treatment and *B. cinerea* infection (Chito+Inf, red circles) were able to induce the differential expression of 543, 2,011 and 2,967 probes at 6, 9 and 12 hpi respectively, of which 260, 991 and 723 DEGs at 6, 9 and 12 hpi respectively were induced only in the presence of chitosan treatment (Figure 3.5). In contrast, non-treated and infected plants (Inf, blue circles), and hence gene expression caused by *B. cinerea* infection, displayed differential expression of 327, 1,134, and 2,697 genes at 6, 9 and 12 hpi respectively, of which 70, 116 and 501 DEGs at 6, 9 and 12 hpi respectively were differentially expressed only in the absence of chitosan treatment (Figure 3.5).

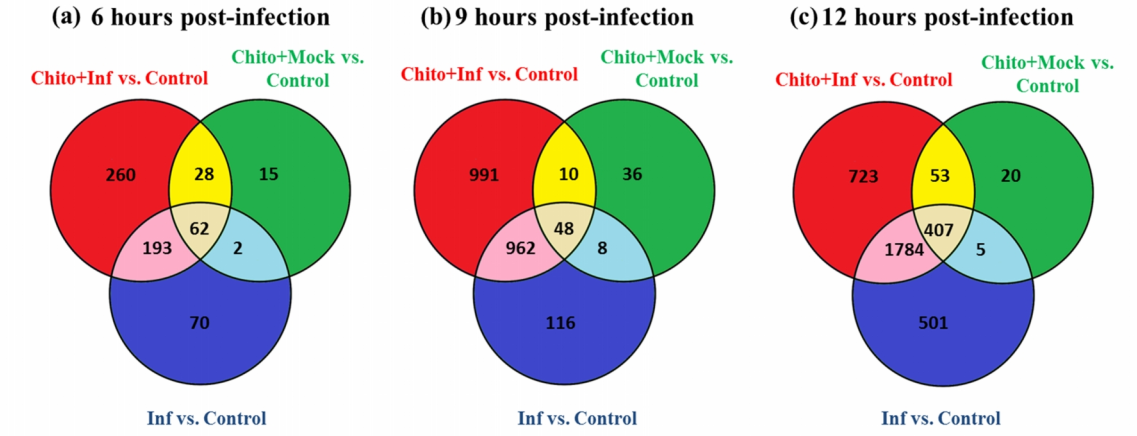


Figure 3.5 Venn diagram illustrating gene expression patterns including the number of common and specific DEGs in Chito+Inf (red circle), Chito+Mock (green circle) and Inf (blue circle) treatments. (**a**) 6 hours post *B. cinerea* BCR16 infection (hpi); (**b**) 9 hours post *B. cinerea* BCR16 infection (hpi); (**c**) 12 hours post *B. cinerea* BCR16 infection (hpi)

Gene lists were extracted from the Venn diagrams in order to identify up/down regulated genes belonging uniquely to Inf (70, 116 and 501 DEGs at 6, 9 and 12 hpi respectively) and Chito+Inf (260, 991 and 723 DEGs at 6, 9 and 12 hpi respectively) for each time point. Overview graphs of differentially expressed genes identified in Venn diagrams showed a high number (1,611 DEGs) of genes down-regulated in Chito+Inf at 6, 9 and 12 hpi, compared to the total of 363 DEGs that were up-regulated (Figures 3.6, 3.7 and 3.8). In contrast, a total 366 DEGs were repressed and 321 DEGs induced in

water-treated and infected (Inf) plants at 6, 9 and 12 hpi (Figures 3.6, 3.7 and 3.8). This clearly shows a greater repression trend of tomato genes as an alternative to activation, in response to *B. cinerea* infection, where chitosan had an impact on the gene repression by increasing the number of genes differentially down-regulated.

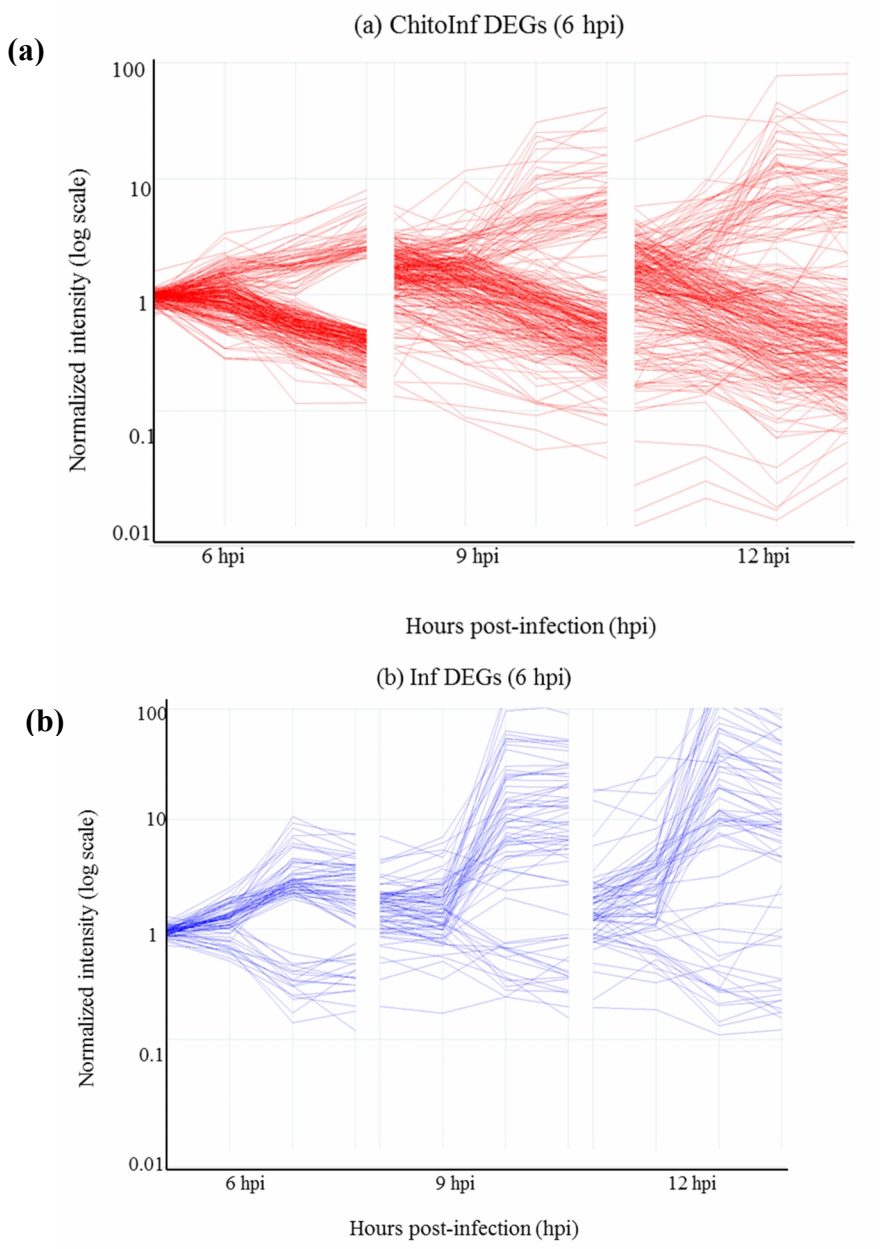


Figure 3.6 Expression patterns overview of differentially up/down-regulated genes identified in Venn diagrams at 6 hpi (**a**) 57 DEGs were up-regulated and 203 DEGs down-regulated uniquely for chitosan-treated + infected treatment (Chito+Inf) (**b**) 52 DEGs were up-regulated and 18 DEGs down-regulated uniquely for non-treated + infected (Inf) treatment.

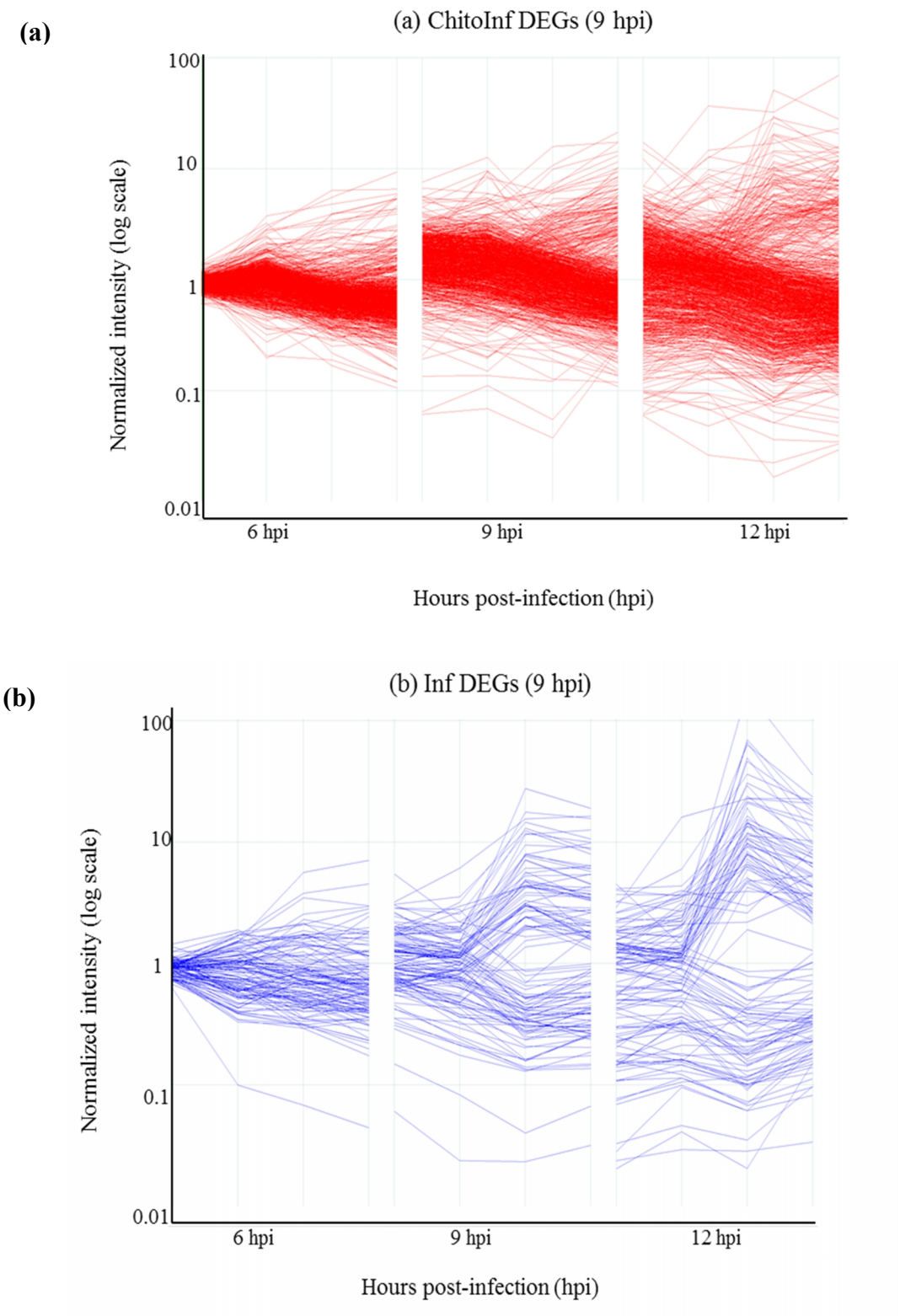


Figure 3.7 Expression patterns overview of differentially up/down-regulated genes identified in Venn diagrams at 9 hpi (**a**) 162 DEGs were up-regulated and 829 DEGs down-regulated uniquely for chitosan-treated + infected treatment (Chito+Inf) (**b**) 55 DEGs were up-regulated and 61 DEGs down-regulated uniquely for water-treated + infected (Inf) treatment.

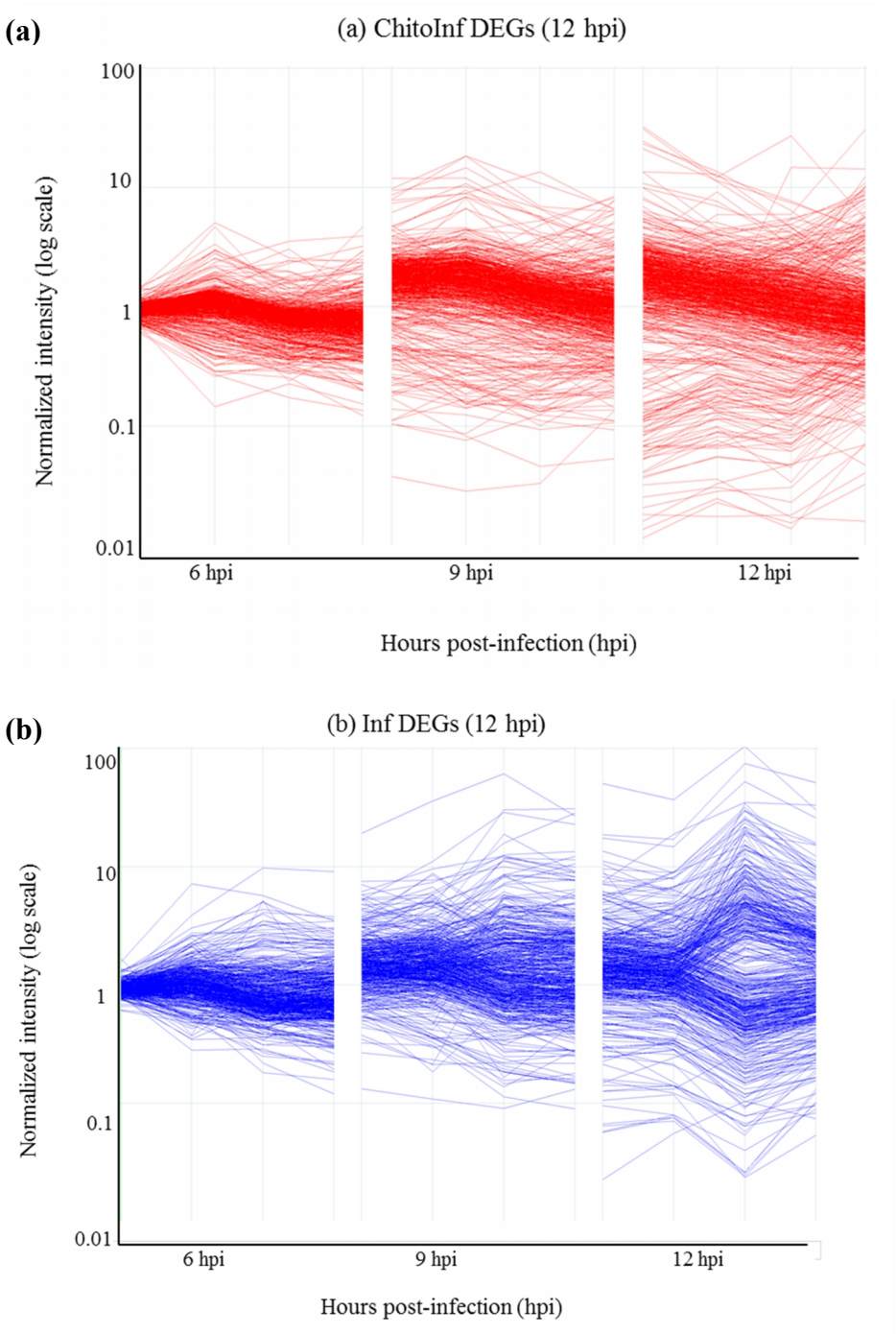


Figure 3.8 Expression patterns overview of differentially up/down-regulated genes identified in Venn diagrams at 12 hpi (**a**) 144 DEGs were up-regulated and 579 DEGs down-regulated uniquely for chitosan-treated + infected treatment (Chito+Inf) (b) 214 DEGs were up-regulated and 287 DEGs down-regulated uniquely for water-treated + infected (Inf) treatment.

3.3.2.2 Identification of significant pathways in chitosan-primed and infected tomatoes (Chito+Inf)

This large-scale transcriptomic analysis helps to identify molecular pathways that might be crucial for chitosan-induced resistance in tomato against *B. cinerea* in the early stages of infection. Venn diagrams revealed that chitosan (Chito+Inf) does prime tomato for a faster and more robust response against *B. cinerea*, differentially expressing 260, 991 and 723 DEGs at 6, 9 and 12 hpi respectively, whereas non-treated and infected plants (Inf) are only able to differentially express 70, 116 and 501 genes at 6, 9 and 12 hpi respectively, in the absence of chitosan (Figure 3.5). Moreover, chitosan down-regulates a higher number of DEGs, in comparison with up-regulated transcripts, during the three asymptomatic stages (6, 9 and 12 hpi) of *B. cinerea* infection (Figures 3.6, 3.7 and 3.8).

3.3.2.2.1 Identification of molecular pathways involved in chitosan-primed tomatoes

To identify molecular pathways involved in chitosan-priming for resistance against *B. cinerea*, the biological functions of DEGs were investigated using bioinformatics software MapMan (Thimm et al. 2004)) by comparing DEGs from Chito+Inf and Inf DEGs, for each time point. Thus, in order to investigate which pathways might be affected by chitosan, DEGs from the Venn diagram lists of genes (Figures 3.5, 3.6, 3.7 and 3.8) from chitosan-treated + infected (Chito+Inf) treatment were compared to water-treated + infected (Inf) per time point with MapMan software.

Significantly repressed pathways by Chito+Inf at 6 hpi included redox state, lipid metabolism, secondary metabolites, hydrolases, ethylene and ERF transcriptional factors. Redox state genes, such as Glutaredoxin (Solyc06g0087501), were found to be significantly down-regulated (blue squares) from Chito+Inf treatment at 6 hpi. Moreover, Chito+Inf resulted in down-regulation (blue) of hydrolase genes compared to an up-regulation (red) in Inf plants (Figure 3.9). Transcriptional factors (ERF, WRKYs and MYB) were significantly induced by Chito+Inf at 6, 9 and 12 hpi whereas Inf resulted in their induction at 12 hpi (Figures 3.9, 3.10, 3.11 and 3.12). Chito+Inf resulted in a down-regulation of Auxin and ethylene-related genes at 6hpi (Figure 3.9) and an induction of cell-wall genes at 6, 9 and 12 hpi compared to a later induction by Inf at 12 hpi. PR-proteins were significantly induced by Chito+Inf at 6, 9 and 12 hpi whereas Inf treatment did not significantly induce PR-proteins in chitosan absence.

Proteolysis and signalling processes (receptor kinases and calcium-dependent protein kinases) were strongly induced by Chito+Inf at 6, 9 and 12 hpi whilst Inf treated plants induction of these processes was weaker (Figures 3.9, 3.10, 3.11 and 3.12). A large number of DEGs, for both Inf and Chito+Inf, were unassigned to any category.

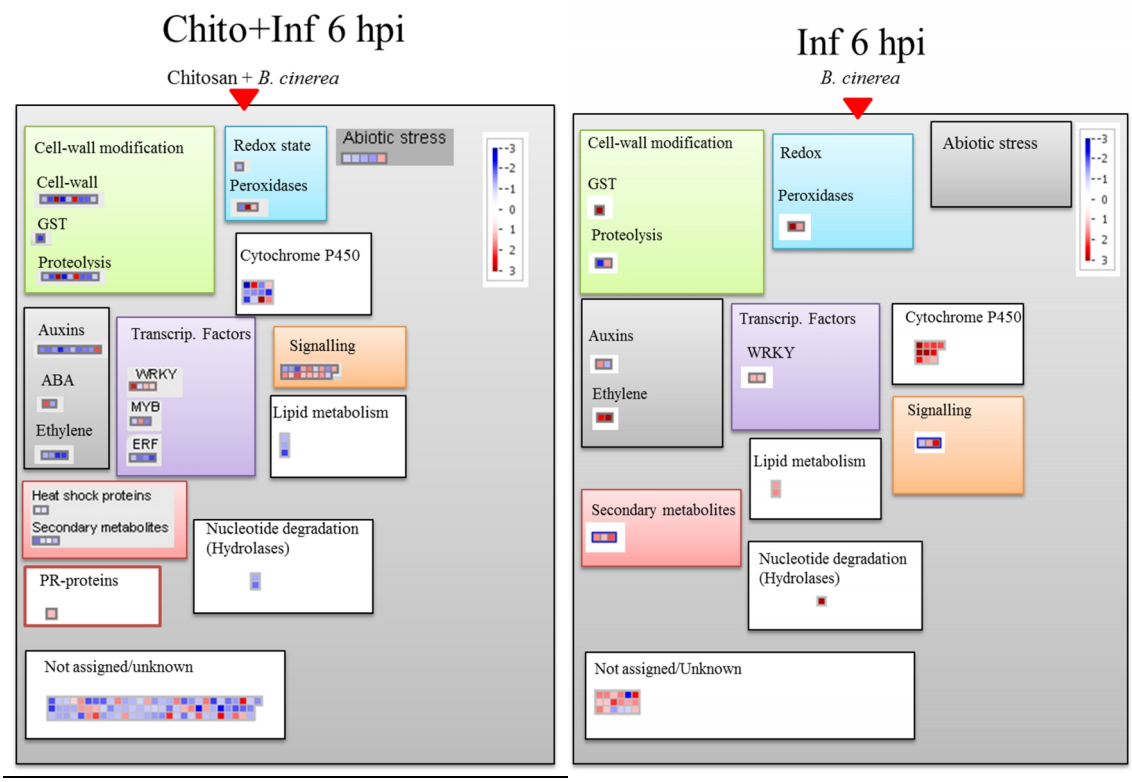


Figure 3.9 Custom diagram of biotic stress and metabolic pathways up/down-regulated inside the cell in chitosan-treated + infected (Chito+Inf) and water-treated + infected (Inf) plants at 6 hpi. 260 DEGs were plotted into MapMan for Chito+Inf and 70 DEGs for Inf treatments from Venn diagram lists. In red are the up- regulated and in blue are the down-regulated genes after *B. cinerea* infection.

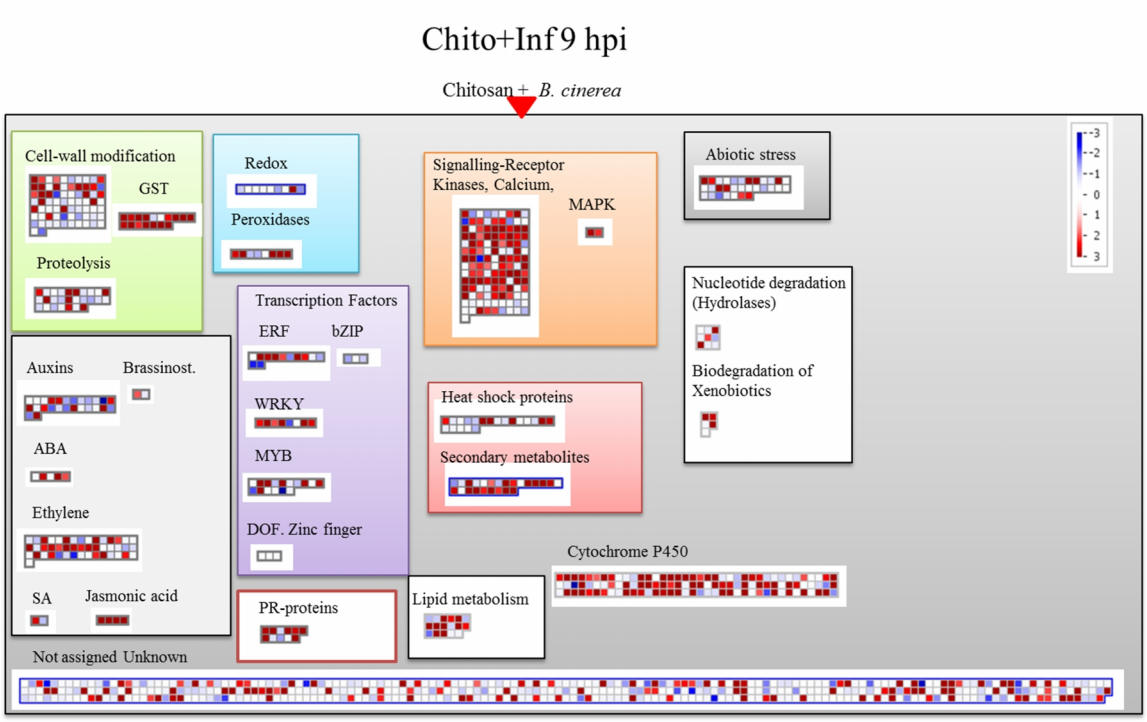


Figure 3.10 Custom diagram of biotic stress and metabolic pathways up/down-regulated inside the cell in chitosan-treated + infection (Chito+Inf) at 9 hpi. 991 DEGs were plotted into MapMan for Chito+Inf from Venn diagram lists. In red are the up- regulated and in blue are the down-regulated genes after *B. cinerea* infection.

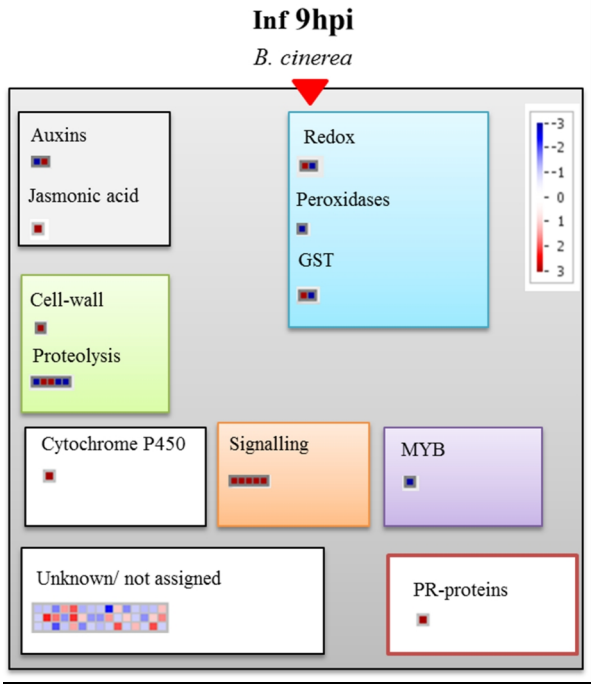


Figure 3.11 Custom diagram of biotic stress and metabolic pathways up/down-regulated inside the cell in water-treated + infection (Inf) at 9 hpi. 116 DEGs were plotted into MapMan for Inf from Venn diagram lists. In red are the up- regulated and in blue are the down-regulated genes after *B. cinerea* infection.

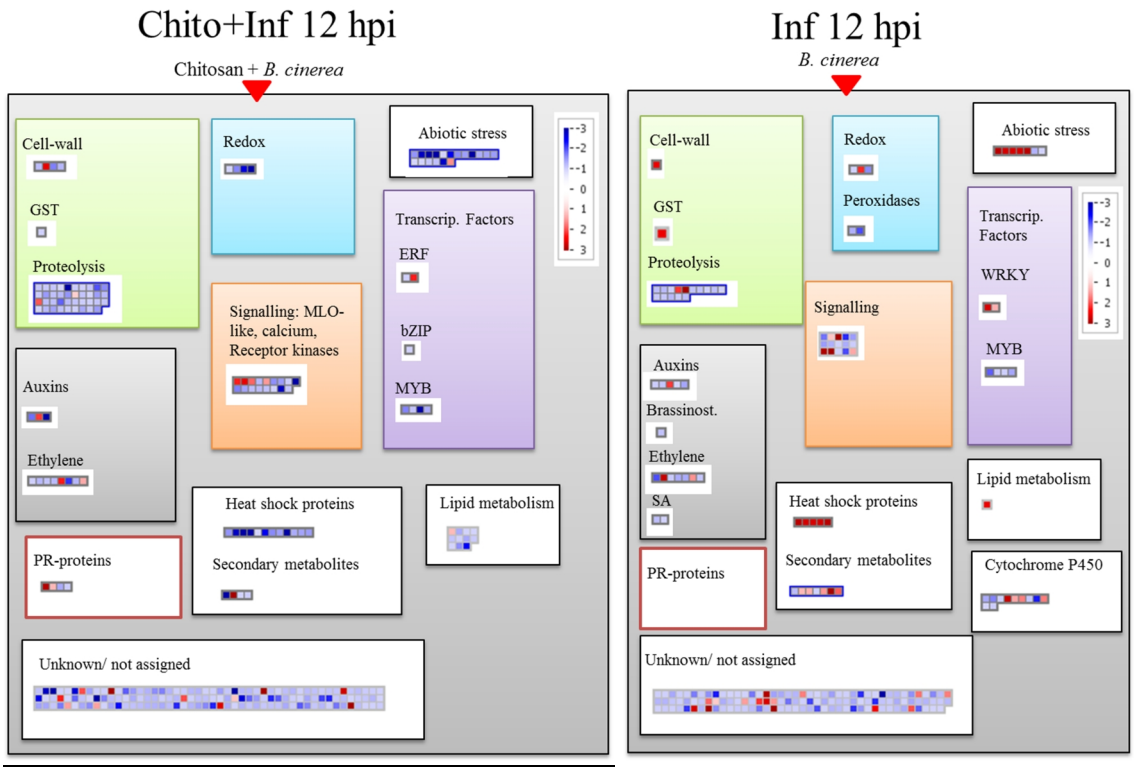


Figure 3.12 Custom diagram of biotic stress and metabolic pathways up/down-regulated inside the cell in chitosan-treated + infected (Chito+Inf) and water-treated + infected (Inf) plants at 12 hpi. 723 DEGs were plotted into MapMan for Chito+Inf and 501 DEGs for Inf treatments from Venn diagram lists. In red are the up- regulated and in blue are the down-regulated genes after *B. cinerea* infection.

3.3.2.2.2 Functional study of gene ontology (GO) terms and gene set enrichment

MapMan analysis has indicated pathways that were uniquely triggered by Chito+Inf and Inf treatments. However, this analysis does not identify enriched functional categories compared to the levels found in the whole tomato genome. Thus, to further investigate which classes of genes are over-represented within the full data set, functional enrichment of differentially expressed genes was analyzed by singular enrichment analysis (SEA) with Panther software (Thomas 2003). This analysis may help us to further identify molecular pathways that may have an association with chitosan-induced resistance phenotype. Here we used chitosan-treated + infected (Chito+Inf) with water-treated + infected (Inf) DEGS at 6, 9 and 12 hpi (lists from Venn diagrams generated with GeneSpring, Figure 3.5) and compared functional categories to those in the whole tomato genome.

GO biological process complete	*S. lycopersicum* ref. #	Upload #	Expected	Fold Enrichment	+/-	P value
response to auxin	209	10	1.58	6.35	+	9.47E-03
response to chemical	916	20	6.91	2.9	+	4.55E-02
response to stimulus	2657	44	20.03	2.2	+	1.25E-03
Unclassified	17617	117	132.83	0.88	-	0.00E+00
GO molecular function complete	***S. lycopersicum* ref. #**	**Upload #**	**Expected**	**Fold Enrichment**	**+/-**	**P value**
cysteine-type peptidase activity	271	11	2.04	5.38	+	1.23E-02
transcription factor activity, sequence-specific DNA binding	856	19	6.45	2.94	+	4.78E-02
nucleic acid binding transcription factor activity	856	19	6.45	2.94	+	4.78E-02
Unclassified	16331	109	123.14	0.89	-	0.00E+00

Figure 3.13 Overrepresentation test (+) of significantly enriched GO terms with Panther for chitosan-treated + infected (Chito+Inf) at 6 hpi, compared to that of the whole tomato genome. Data was loaded into Panther tool and statistical analysis ($P<0.05$) with Bonferroni correction for multiple testing was performed.

GO biological process complete	*S. lycopersicum* ref. #	Upload #	Expected	Fold Enrichment	+/-	P value
ornithine metabolic process	20	3	0.04	72.75	+	1.86E-02
Unclassified	17617	29	36.32	0.8	-	0.00E+00

Figure 3.14 Overrepresentation test (+) of significantly enriched GO terms with Panther for non-treated + infected (Inf) at 6 hpi, compared to that of the whole tomato genome. Data was loaded into Panther tool and statistical analysis ($P<0.05$) with Bonferroni correction for multiple testing was performed.

Functional enrichment of DEGs belonging to the two infected conditions (Inf and Chito+Inf) revealed various biological processes that were significantly enriched for Chito+Inf at 6 hpi (Figure 3.13). Response to stimulus and chemical genes were

significantly overrepresented, including cell-wall-associated receptor kinases, Mitogen-activated protein kinases (MAPKs), gluthatione-S-transferases (GST), Auxin-related genes, Gibberellin 2-oxidases, peroxidases and Chlorophyll a-b binding proteins (Figure 3.13).

Moreover, 19 transcriptional factors were significantly overrepresented, such as genes belonging to APETALA2/Ethylene-responsive element binding protein family, ethylene responsive transcription factors, WRKYs and MYBs. For water-treated + infected (Inf) plants, only ornithine metabolic process was significantly enriched at 6 hpi, including 2 N-hydroxycinnamoyl-CoA:tyramine N-hydroxycinnamoyl transferases (Figure 3.14).

GO biological process complete	***S. lycopersicum* ref. #**	**Upload #**	**Expected**	**Fold Enrichment**	**+/-**	**P value**
intracellular signal transduction	468	32	13.55	2.36	+	2.00E-02
signal transduction	1145	66	33.15	1.99	+	3.13E-04
response to stimulus	2657	116	76.93	1.51	+	1.43E-02
signaling	1145	66	33.15	1.99	+	3.13E-04
regulation of cellular process	3627	156	105.01	1.49	+	7.46E-04
regulation of biological process	3914	165	113.32	1.46	+	1.10E-03
biological regulation	4531	184	131.18	1.4	+	2.44E-03
cell communication	1233	71	35.7	1.99	+	1.10E-04
protein phosphorylation	1232	75	35.67	2.1	+	4.83E-06
phosphorylation	1549	83	44.85	1.85	+	1.73E-04
phosphate-containing compound metabolic process	2181	104	63.15	1.65	+	1.05E-03
phosphorus metabolic process	2228	104	64.51	1.61	+	2.70E-03
cellular protein modification process	2644	118	76.55	1.54	+	4.34E-03
protein modification process	2644	118	76.55	1.54	+	4.34E-03
macromolecule modification	3287	135	95.17	1.42	+	4.72E-02
Unclassified	17617	466	510.06	0.91	-	0.00E+00
GO molecular function complete	***S. lycopersicum* ref. #**	**Upload #**	**Expected**	**Fold Enrichment**	**+/-**	**P value**
signaling receptor activity	255	21	7.38	2.84	+	3.98E-02
signal transducer	380	28	11	2.54	+	1.54E-02

activity						
protein serine/threonine kinase activity	820	53	23.74	2.23	+	1.45E-04
protein kinase activity	1230	75	35.61	2.11	+	3.71E-06
kinase activity	1450	81	41.98	1.93	+	3.72E-05
transferase activity, transferring phosphorus-containing groups	1675	86	48.5	1.77	+	4.87E-04
catalytic activity, acting on a protein	2945	140	85.27	1.64	+	8.90E-06
phosphotransferase activity, alcohol group as acceptor	1362	78	39.43	1.98	+	2.48E-05
Unclassified	16331	426	472.83	0.9	-	0.00E+00
structural constituent of ribosome	458	1	13.26	< 0.2	-	3.23E-02
structural molecule activity	545	2	15.78	< 0.2	-	2.53E-02

Figure 3.15 Overrepresentation test (+) of enriched GO terms with Panther for chitosan-treated + infected (Chito+Inf) at 9 hpi. Data was loaded into Panther tool and statistical analysis ($P<0.05$) with Bonferroni correction for multiple testing was performed.

Functional enrichment study of DEGs belonging to the two infected conditions (Inf and Chito+Inf) at 9 hpi revealed 15 enriched biological processes and 8 molecular pathways for Chito+Inf plants, including intracellular signalling and protein phosphorylation as the main significantly overrepresented biological processes. Enriched signalling genes included numerous cell-wall associated serine/threonine-protein kinases involved in protein phosphorylation and other protein modification processes; mitogen-activated protein kinases (MAPKs) and ethylene-responsive transcriptional factors. Moreover, other examples of over-repressented genes included Auxin-related genes, Gibberellin 2-oxidases, MYB, basic helix-loop-helix (bHLH), AP2-like ethylene-responsive, WRKY transcription factors, B-Box zinc finger proteins and thioredoxins (Figure 3.15). In contrast, water-treated + infected plants did not have any enriched pathways at 9 hpi. At 12 hpi, Chito+Inf showed 1 enriched pathway, photosynthesis-light reaction proteins; whilst Inf treatment fructose metabolic pathway enriched pathways (Figures 3.16 and 3.17).

GO biological process complete	*S. lycopersicum* ref. #	Upload #	Expected	Fold Enrichment	+/-	P value
photosynthesis, light reaction	102	13	2.15	6.04	+	7.39E-04

Figure 3.16 Overrepresentation test (+) of enriched GO terms with Panther for chitosan-treated + infected (Chito+Inf) at 12 hpi. Data was loaded into Panther tool and statistical analysis (P<0.05) with Bonferroni correction for multiple testing was performed.

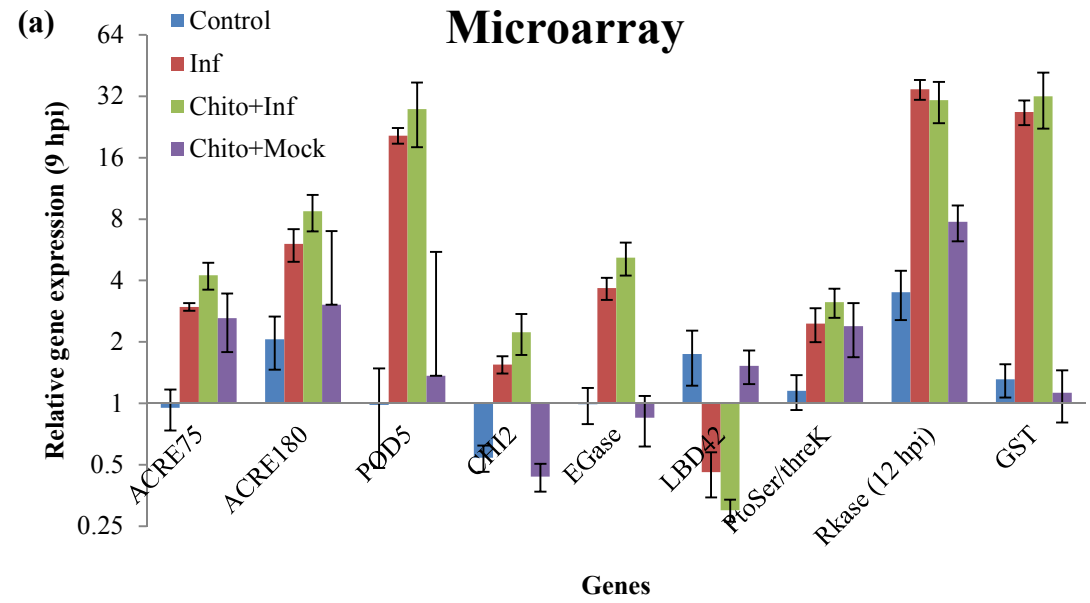
GO biological process complete	*S. lycopersicum* ref. #	Upload #	Expected	Fold Enrichment	+/-	P value
fructose metabolic process	8	4	0.11	35	+	1.10E-02

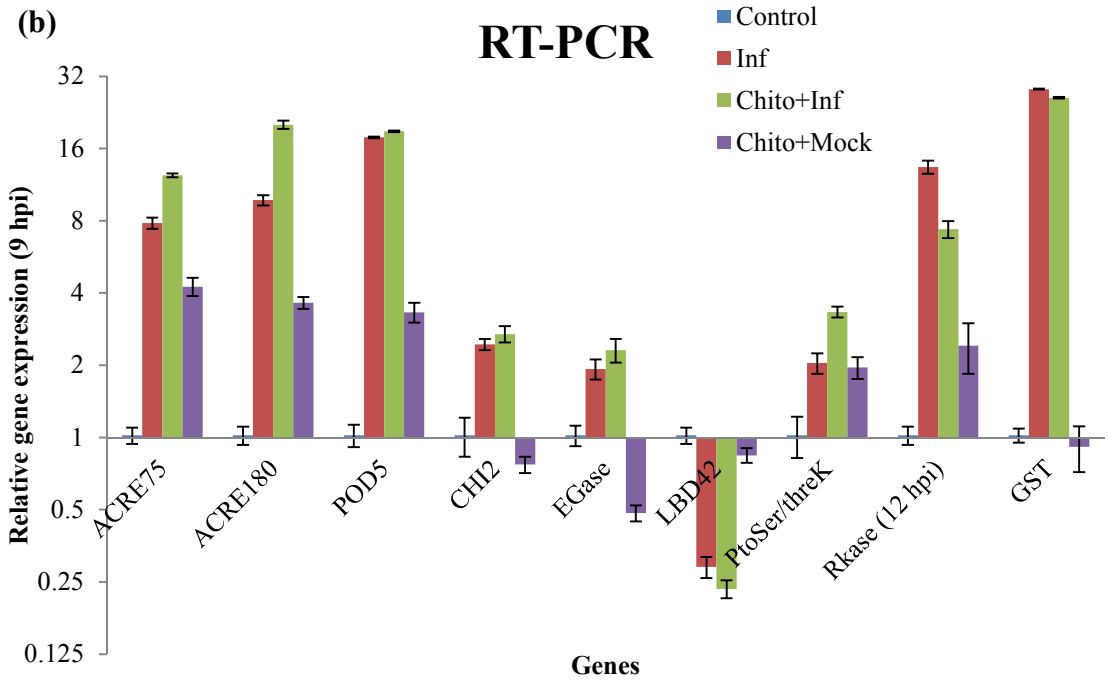
Figure 3.17 Overrepresentation test (+) of significantly enriched GO terms with Panther for non-treated + infected (Inf) at 12 hpi. Data was loaded into Panther tool and statistical analysis (P<0.05) with Bonferroni correction for multiple testing was performed.

3.3.2.3 Characterisation of specific gene candidates involved in chitosan-priming for resistance tomato plants against *Botrytis cinerea*

Functional enrichment analysis helped understand chitosan priming mechanisms on tomato plants against fungal necrotroph *B. cinerea*, by inducing a more robust and earlier set of genes (Figures 3.5, 3.6, 3.7 and 3.8) and by significantly over-representing (enriching) key defence pathways to fight *B. cinerea* infection (Figures 3.13, 3.14, 3.15, 3.16 and 3.17). However, analysis of large transcriptional data sets with software tools to identify metabolic pathways or other molecular processes (i.e. MapMan, Panther or gProfiler) can filter important genes whose functions are not yet assigned or well-characterised (Figures 3.9, 3.10, 3.11 and 3.12). For this reason, genes that showed a 'priming phenotype' by being significantly expressed (ANOVA-BH) earlier and/or stronger (e.g. a high level of up/down regulation) during the infection on chitosan-primed and infected plants (Chito+Inf) (Venn diagrams, Figure 3.5 and Figure 3.18c) were investigated in more detail.

A list of candidate differentially expressed genes (DEGs) belonging to different pathways were selected on the basis of their function, and expression patterns of the candidate genes were quantified by an alternative method (RT-qPCR). The fold-change expression levels of the candidate DEGs studied by RT-qPCR behaved similarly to the expression studied by microarray hybridization, which had the benefit of validating the array data (Figure 3.18). Candidate DEGs include ROS-type defence genes, such as peroxidase 5; cell-wall-related enzyme endo-1,3-β-glucanase (EGase) gene; pathogenesis-related chitinase (CHI) 2; also a novel transcriptional factor family involved in pathogenicity, such as LATERAL ORGAN BOUNDARIES (LOB) DOMAIN (LBD)-CONTAINING PROTEIN42 (LBD42) and genes involved in signalling after pathogen challenge such as Pto-like, Serine/threonine kinase protein, resistance protein and receptor serine/threonine kinase-like protein. Unassigned categories included some notable DEGs with a strong priming phenotype, such as Avr9/CF9 rapid elicit (ACRE) gene 75, which molecular function is to date unknown (discussed in more detail in Chapter 4).


(a)
Microarray
Control
Inf
Chito+Inf
Chito+Mock
Relative gene expression (9 hpi)
64
32
16
8
4
2
1
0.5
0.25
ACRE75
ACRE180
POD5
CHI2
EGase
LBD42
PtoSer/threK
Rkase (12 hpi)
GST
Genes


(b)
RT-PCR
Control
Inf
Chito+Inf
Chito+Mock
Relative gene expression (9 hpi)
32
16
8
4
2
1
0.5
0.25
0.125
ACRE75
ACRE180
POD5
CHI2
EGase
LBD42
PtoSer/threK
Rkase (12 hpi)
GST
Genes

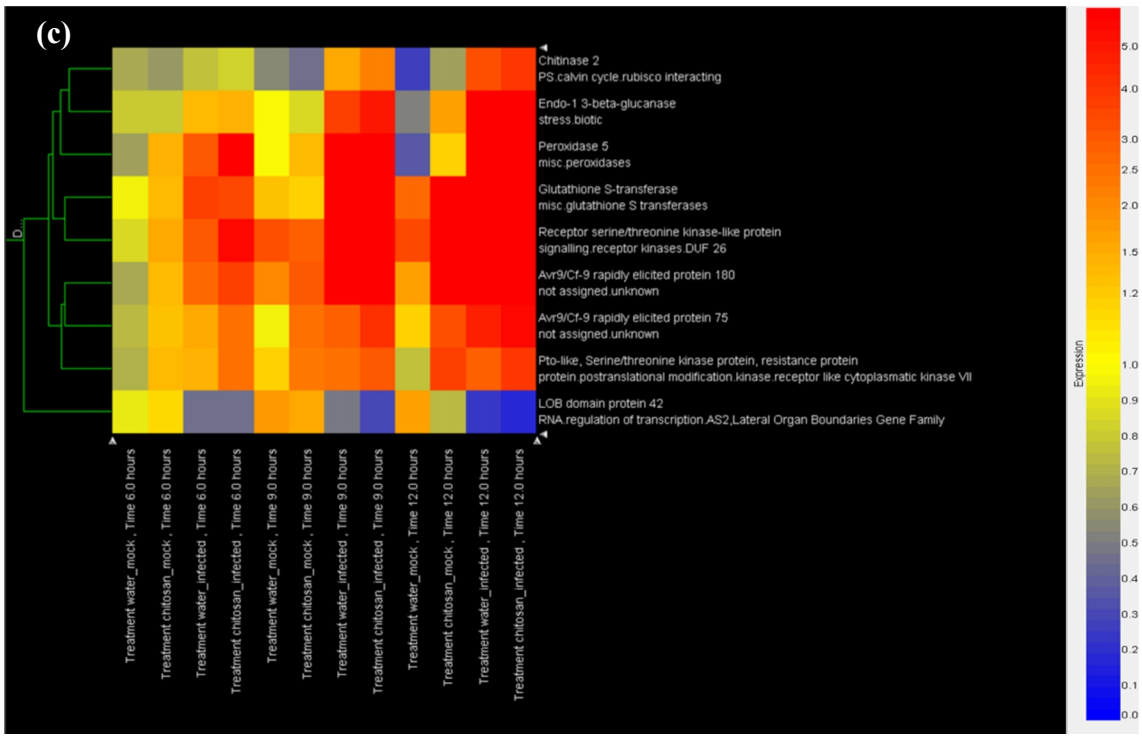


Figure 3.18 Gene expression for a selected number of genes at 9 hpi, as measured by **(a)** Fold change (Log2) normalized values obtained by microarray hybridization. **(b)** Fold change (Log2) values obtained by reverse transcription-quantitative polymerase chain reaction (qRT-PCR) against two reference genes (actin and ubiquitin) and **(c)** Heatmap of candidate genes identified in microarray analyses from the ANOVA-BH. Hierarchical cluster analysis in GeneSpring software. The colour scale represents gene up-regulation (red), no change in expression (yellow) and down-regulation (blue). Every column represents a different treatment at the 3 time points (6, 9 and 12 hpi). The data shown are the means of three biological replicates for the qRT-PCR and 4 biological replicates for the Microarray ± standard error of the mean (SEM). Relative Quantitation (RQ) values were compared after normalization (efficiency validation) to actin and ubiquitin expression levels. Control, ddH_2O-treated and non-infected; Inf, ddH_2O-treated and *B. cinerea*-infected plants; Chito+Mock, chitosan-treated and non-infected plants; Chito+Inf, chitosan-treated and infected plants at 9 hpi. ACRE75, ACRE180, Avr9/Cf-9 rapidly elicited protein 75/180; POD5, Peroxidase 5; CHI2, chitinase2; LBD42, lateral organ boundaries domain protein 42, PtoSer/threK, Pto-like, Serine/threonine kinase protein, resistance protein; RKase, Receptor serine/threonine kinase-like protein (at 12 hpi), GST, Glutathione S-transferase.

3.3.2.4 Characterisation of specific molecular pathways involved in chitosan-priming for resistance against *Botrytis cinerea* in *Solanum lycopersicum*

Two functional groups of interest from the identified pathways (GO) analysis were assessed in further detail, for cell wall and hormone signalling.

3.3.2.4.1 Identification of cell-wall candidate genes involved in chitosan-priming resistance against *Botrytis cinerea* in *Solanum lycopersicum*

Transcriptomic analysis has revealed key molecular pathways induced by chitosan in tomato to fight back *B. cinerea* early stages of infection, such as cell-wall related genes, signalling and receptor-like kinase cascades, secondary metabolites, hormone-related transcription factors and redox processes. In particular, cell-wall related genes, such as receptor-like kinases (RLKs) were significantly triggered by Chito+Inf earlier and with a stronger representation during the infection (6 and 9 hpi) and they were over-represented at 6 and 9 hpi in Chito+Inf (Figures 3.5, 3.9, 3.10, 3.12, 3.13 and 3.15). This suggests a role of chitosan in priming cell-wall processes to fight *B. cinerea* infection. Previous results demonstrated that chitosan may prime callose deposition in tomato epidermal cells when applied at low-concentrations, as the lowest concentration (0.001 % w/v) induced callose strongly and for a longer period than higher concentrations of 0.01 and 0.1 % w/v (Figure 2.16).

The cell-wall constitutes the very first plant barrier against fungal invading pathogens. Therefore, it is logical to investigate whether chitosan can prime cell-wall dependent genes. Thus, in order to investigate cell-wall processes, cluster analysis was performed for all cell-wall dependent genes from the comparative analysis ANOVA. A total of 40 DEGs were found split into three main clusters (Figure 3.19).

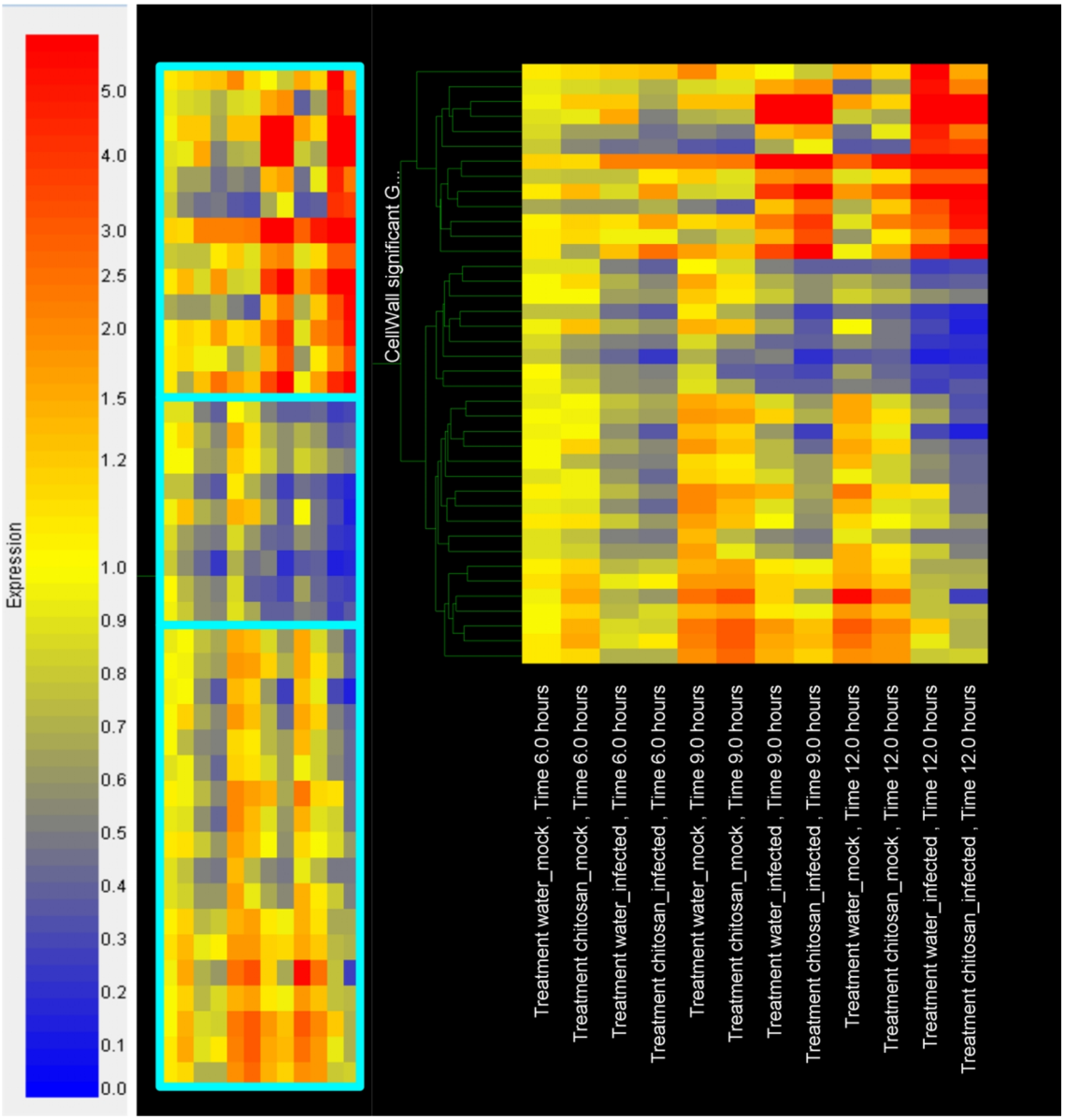


Figure 3.19 Overview of differentially expressed cell-wall candidate genes (DEGs) identified in microarray analysis using ANOVA-BH ($P \leq 0.05$). 40 cell-wall DEGs were found, divided into 3 clusters (blue rectangles). Hierarchical cluster analysis in GeneSpring software. The colour scale represents gene up-regulation (red), no change in expression (yellow) and down-regulation (blue). Blue boxes represent 3 main clusters found. Every column represents a different treatment at the 3 time points (6, 9 and 12 hpi); (i) water_Mock (Control); (ii) chitosan_mock (Chito+Mock); (iii) water_infected (Inf); (iv) chitosan_infected (Chito+Inf).

The first cluster comprising up-regulated DEGs shows genes involved in cellulose synthesis, such as COBRA-like protein and bifunctional polymyxin resistance protein (ArnA), being strongly induced by chitosan-treated + infection (Chito+Inf) earlier at 6, 9 and 12 hpi whereas water-treated + infection (Inf) treatment only induced them later

and weakly at 9 and 12 hpi (Figure 3.20). However, some endoglucanase genes were induced higher by Inf.

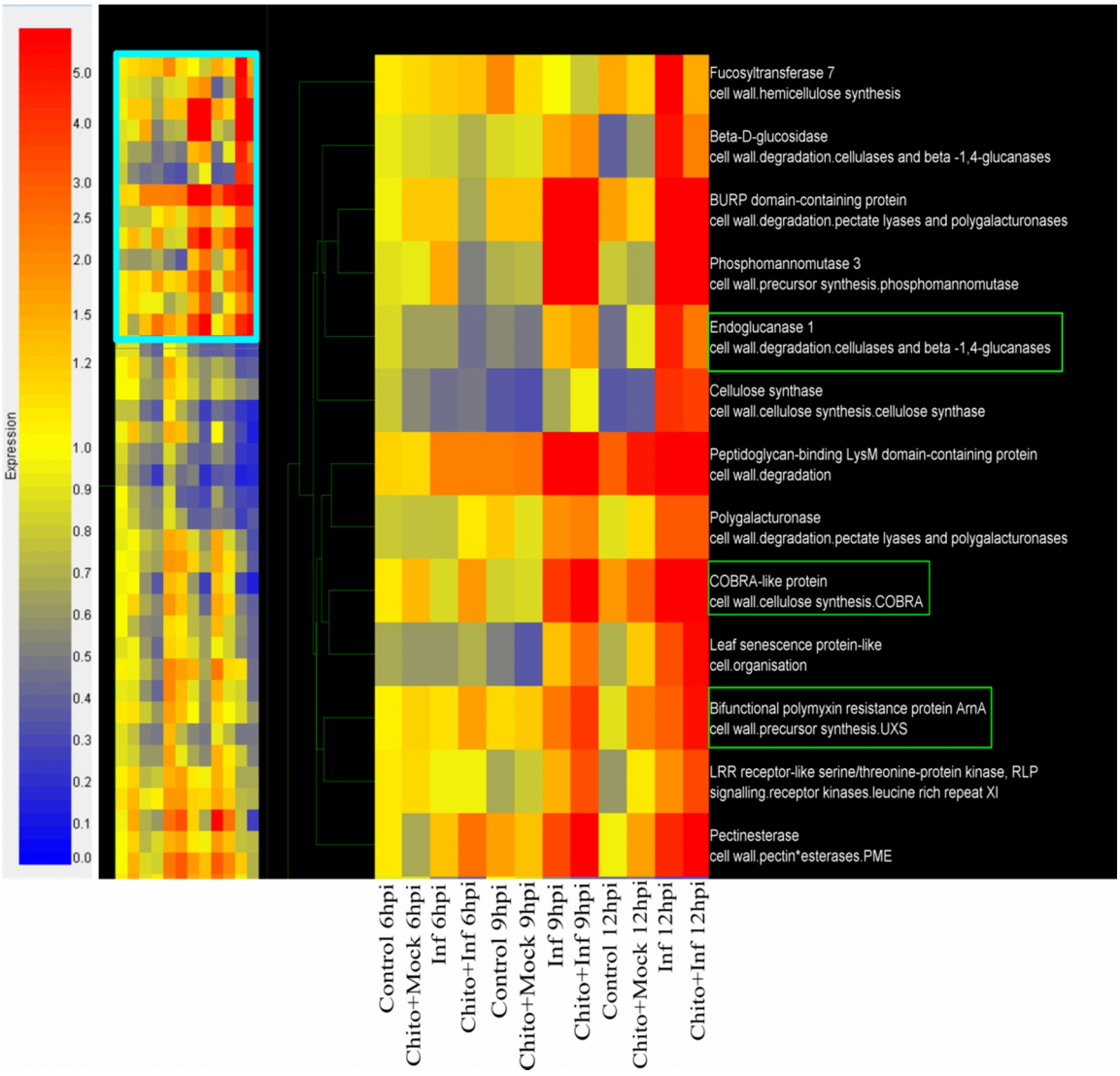


Figure 3.20 Overview of differentially expressed cell-wall candidate genes (DEGs) identified in microarray analysis using ANOVA-BH ($P \leq 0.05$). 40 cell-wall DEGs were found. Hierarchical cluster analyses in GeneSpring software. The colour scale represents gene up-regulation (red), no expression (yellow) and down-regulation (blue). Blue box represent first main cluster found. Every column represents a different treatment at the 3 time points (6, 9 and 12 hpi).

The second and third clusters contained down-regulated genes showed cell-wall degradation enzymes (hydrolases), such as polygalacturonases and pectinesterases being highly down-regulated by Chito+Inf at 6, 9 and/or 12 hpi whilst Inf plants down-regulation was weaker and/or later (Figure 3.21).

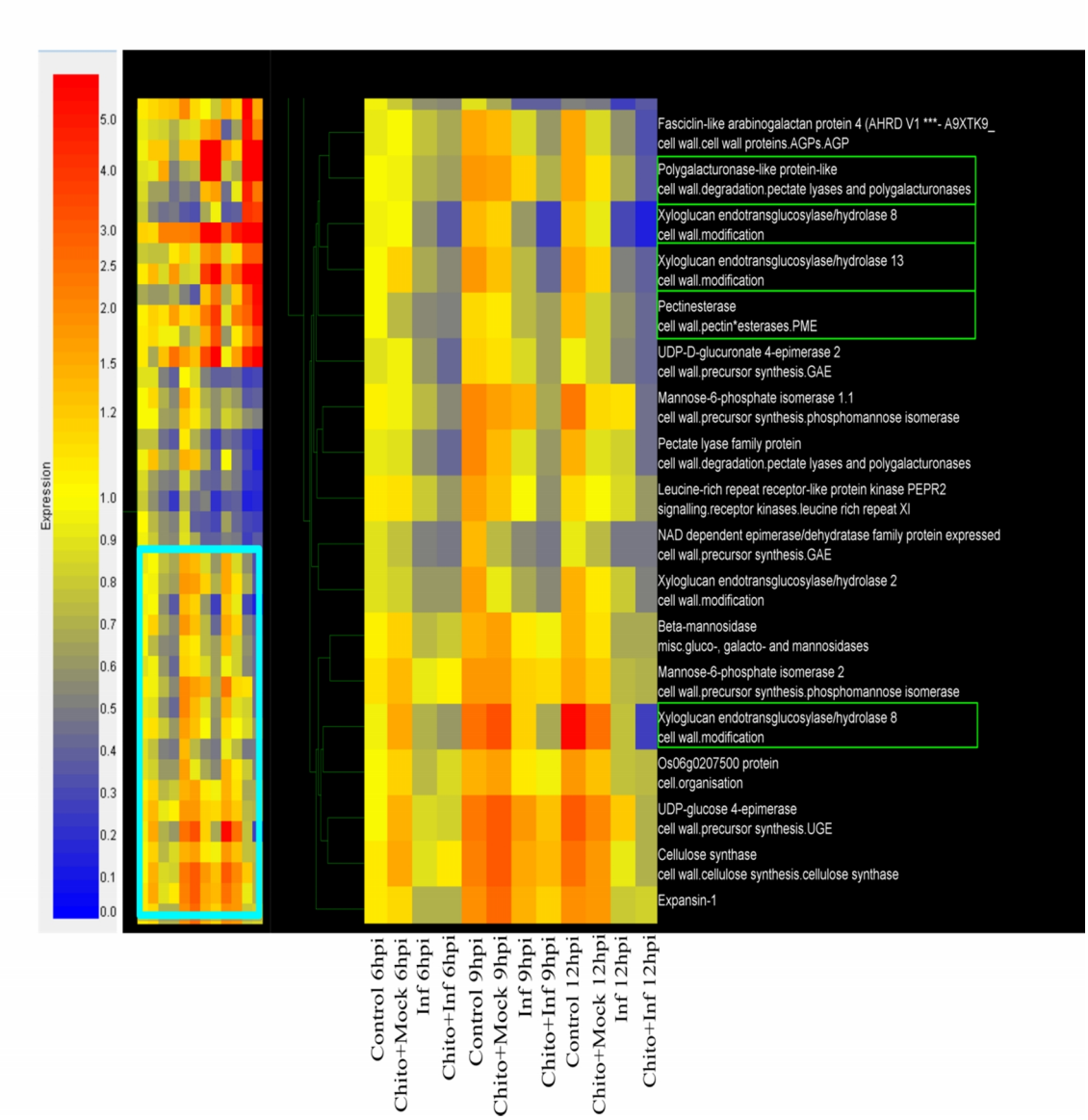


Figure 3.21 Overview of differentially expressed cell-wall candidate genes (DEGs) identified in microarray analysis using the ANOVA-BH ($P \leq 0.05$). 40 cell-wall DEGs were found. Hierarchical cluster analyses in GeneSpring software. The colour scale represents gene up-regulation (red), no expression (yellow) and down-regulation (blue). Blue box represent bottom main cluster found. Every column represents a different treatment at the 3 time points (6, 9 and 12 hpi).

3.3.2.4.2 Identification of phytohormone candidate genes involved in chitosan-priming for resistance against *Botrytis cinerea* in *Solanum lycopersicum*

Whole transcriptome analysis has revealed key molecular pathways involved in chitosan-priming tomato to fight back early stages of *B. cinerea* infection, such as cell-wall modification genes, signalling and redox processes. It is well-accepted by the scientific community that phytohormones play crucial roles in the regulation of the defence signalling network upon perception of biotic or abiotic stress (Forcat et al.

2008; Pieterse et al. 2012). MapMan custom diagrams have revealed stress-related hormone pathways induced by Chito+Inf, such as auxins, ABA, ethylene, brassinosteroids, salicylic acid and jasmonate metabolism (Figures 3.9 and 3.10). Furthermore, hormone pathways were significantly over-represented in Chito+Inf at 6 and 9 hpi, including auxin, gibberellin, jasmonic and ethylene-associated transcriptional factors such as AP2/ERF responsive factors, bHLH and MYBs (Figures 3.13 and 3.15), whereas Inf plants did not significantly enrich any hormonal-related genes or pathways (Figures 3.14, 3.16 and 3.17). Therefore, I concluded to investigate marker hormone-dependent genes that might be primed by chitosan. In order to elucidate role of key hormonal-related genes in chitosan-tomato-*Botrytis cinerea* interaction, differential expression marker genes involved in jasmonic acid (MYC2, JAZ1), ethylene (AP2/ERF), abscisic acid (ABAPYL4) and salicylic acid (NPR1) pathways were investigated.

As shown in the heatmap, basic helix-loop-helix (bHLH) transcriptional factor (TF) (MYC2) was down-regulated at 6 hpi and 9 hpi for both non-treated and infected (Inf) and chitosan-primed and infected (Chito+Inf) treatments (Figure 3.22). However, *SlMYC2* was only down-regulated by Inf at 12 hpi, which suggests a shift in JA-dependent defence from this time point due to chitosan treatment. *SlJAZ1*, a JA-defence pathway repressor, showed an antagonist expression pattern to *SlMYC2*, were it was up-regulated by both Inf and in a higher extent by Chito+Inf at 6 and 9 hpi, however at 12 hpi it was noticed that Chito+Inf-*SlJAZ1* induction was weaker than Inf treatment (Figure 3.22).

To further investigate the well-known JA-SA antagonism, the role of ABA signalling and the synergistic JA/ET cross-talk in plant against necrotrophic pathogens, and therefore to elucidate whether chitosan is able to reduce *B. cinerea* potential defence manipulation, *SlNPR1*, *SlAP2/ERF* and *SlABAPYL4* transcripts expression was measured. *SlNPR1-1*, *SlAP2-like* (ERF) and *SlABAPYL4* were found to be differentially expressed by Chito+Inf treatment at 6, 9 or 12 hpi (ANOVA-BH). *SlNPR1-1* was down-regulated by both Inf and Chito+Inf at 6 hpi (Figure 3.22). However, at 9 and 12 hpi, *SlNPR1-1* expression changed and it was only down-regulated by Chito+Inf whereas water-treated and infected plants (Inf) did not repress it (Figure 3.22). *SlAP2/ERF* was

down-regulated by Inf and Chito+Mock treatments and it was only up-regulated by Chito+Inf at 6, 9 and 12 hpi (Figure 3.22).

Alternatively, *SlABAPYL4* showed the opposite transcriptional response by a stronger down-regulation in Chito+Inf at 6, 9 and 12 hpi in comparison with Inf and Chito+Mock treatments (Figure 3.22).

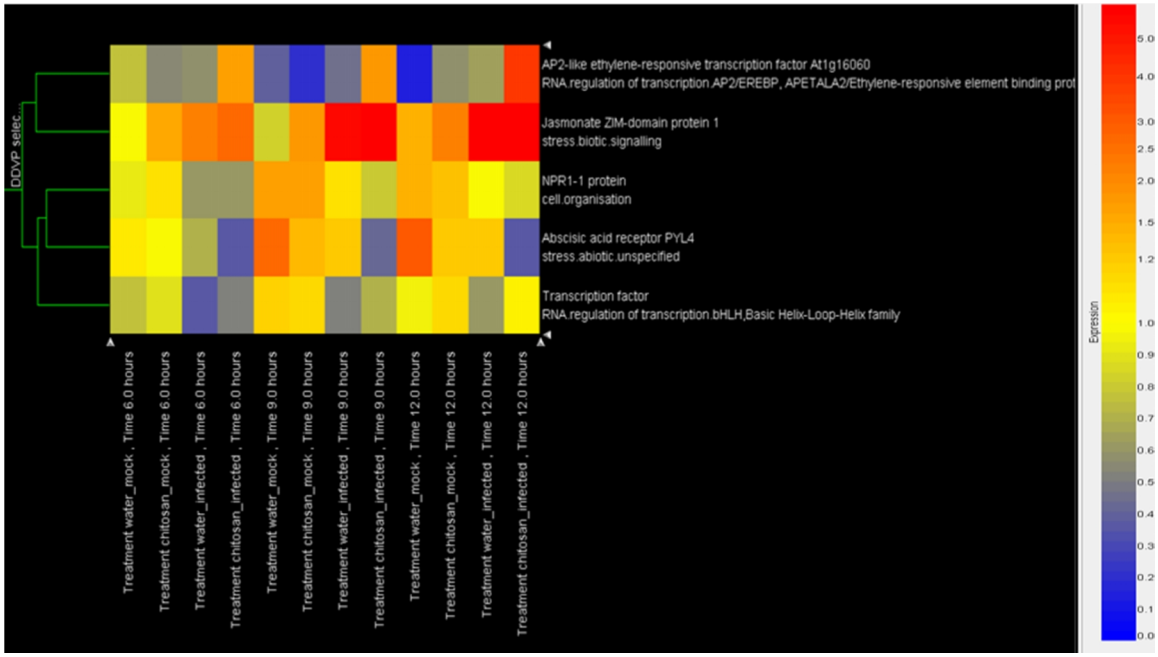


Figure 3.22 Heatmap of hormone-related candidate transcription factors identified in microarray analyses from the ANOVA-BH. From top to bottom: JA/ET-dependent SlAP2-ERF and SlJAZ1; SA-dependent SlNPR1-1; ABA-dependent SlABAPYL4 and JA-dependent Basic helix-loop-helix (bHLH) MYC2. Hierarchical cluster analysis in GeneSpring software. The colour scale represents gene up-regulation (red), no change in expression (yellow) and down-regulation (blue). Every column represents a different treatment at the 3 time points (6, 9 and 12 hpi).

These findings clearly show a priming effect of chitosan in fine-tuning tomato hormone-dependent defences and therefore to improve hormonal cross-talk to ultimately increase tomato resistance to *B. cinerea*.

3.3.3 Transcriptomic analysis of *Botrytis cinerea* in chitosan-primed and non-primed *Solanum lycopersicum*

The primary objective of this transcriptomic study was to identify genes in highly susceptible *S. lycopersicum* cv. Moneymaker involved in chitosan-priming for

resistance to *B. cinerea*. However, one of the main advantages of the experimental set up is the ability to investigate both host and pathogen simultaneously.

This can add valuable information and hence help to decipher the still poorly understood *B. cinerea* complex infection strategy that might help to identify well-known and novel virulence genes involved in the early stages of the infection. This transcriptomic analysis was a joint 60k Agilent array design made with 16,365 *B. cinerea* probes & 34,616 *S. lycopersicum* probes.

3.3.3.1 Identification of differentially expressed *B. cinerea* genes

For the transcriptomic analysis on the *B. cinerea*, the level of pathogen gene expression was low compared to the plant signal, due to early stages of the infection and possibly low amount of fungal biomass in comparison with plant RNA in the samples. This meant some challenges in the statistical analysis and caused that few gene sequences from *B. cinerea* were detected at the 6 h and 9 h time point after inoculation (hpi) with fungal conidia. The study could be improved by using increased numbers of biological replicates, thereby potentially allowing more stringent statistical testing.

However, for the current analysis, the 2,015 probe list (Raw >50, see M&M) was used. Statistical test (T-test pairwise) on water-treated + infected (Inf) and chitosan-treated + infected (Chito+Inf) samples was performed. 3 lists of genes (one per time point) were created from the T-tests pairwise statistical tests:

1. 6 hpi: 21 genes were found differentially expressed from the comparison of water-treated + infected (Inf) with chitosan-treated + infected (Chito+Inf) at 6 hpi
2. 9 hpi: 20 genes were found differentially expressed from the comparison of water-treated + infected (Inf) with chitosan-treated + infected (Chito+Inf) at 9 hpi
3. 12 hpi: 321 genes were found differentially expressed from the comparison of water-treated + infected (Inf) with chitosan-treated + infected (Chito+Inf) at 12 hpi

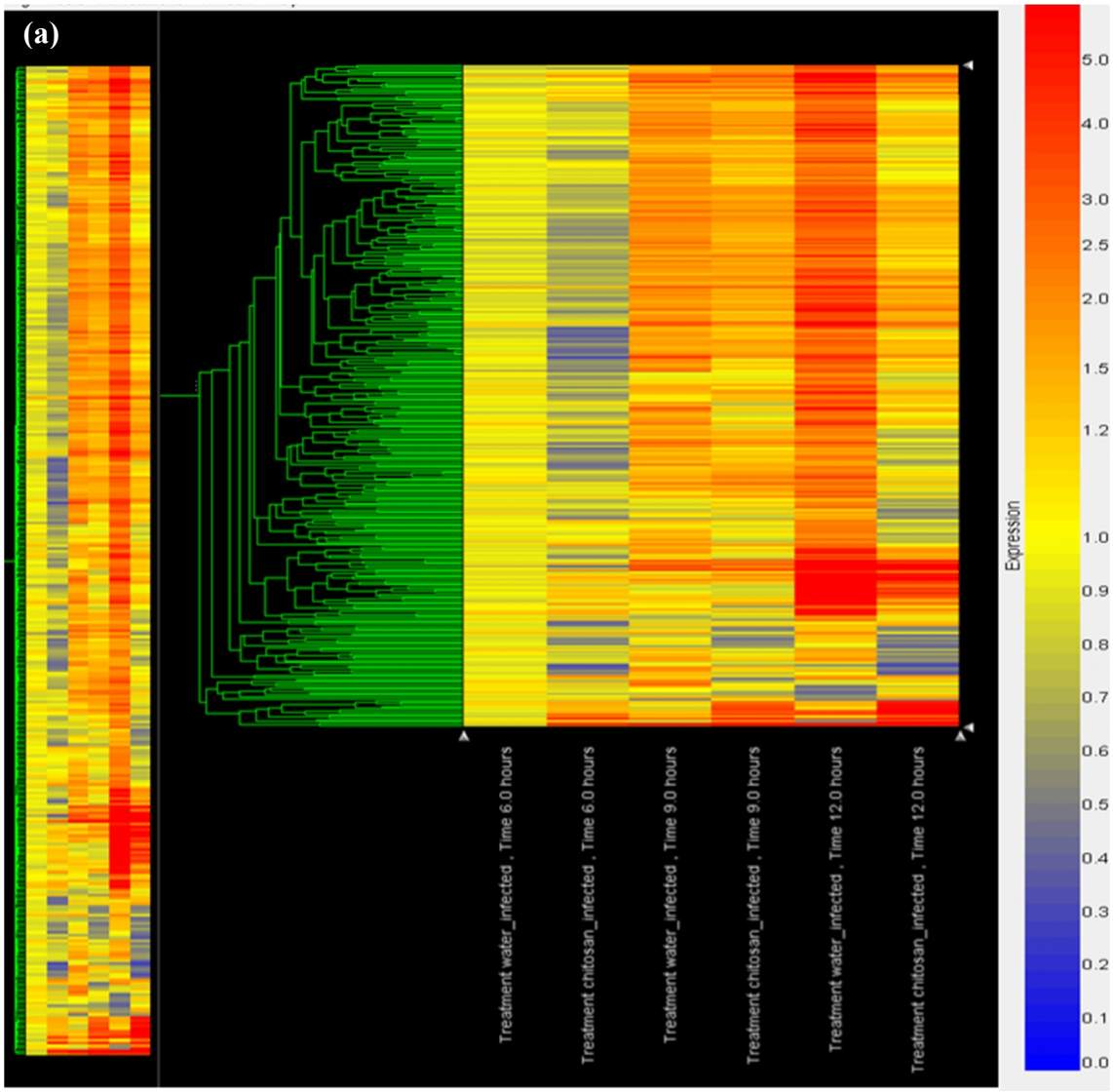
Therefore, specific genes from the 3 lists were selected for gene expression analysis and literature search was performed in order to find whether they have been described in previous studies.

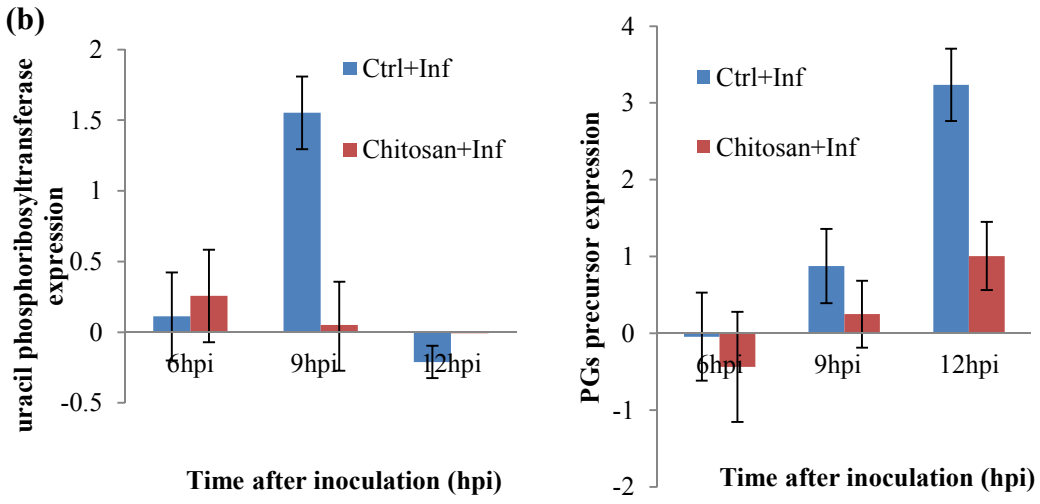
3.3.3.2 *Botrytis cinerea* virulence genes expression is repressed in chitosan-primed tomatoes

Heatmap showed a strong repression of *B. cinerea* genes by Chito+Inf treatment at 6 hpi, some of the genes continued being repressed by chitosan during the infection (Figure 3.23a).

Some examples include hexokinase, which showed a faster and stronger expression in Inf whereas Chito+Inf showed weaker expression (Figure 3.23b).

B. cinerea superoxide dismutase (SOD) was only up-regulated at 9 and 12 hpi in Inf plants whereas Chito+Inf repressed its expression (Figure 3.23b). Uracil phosphoribosyltransferase (*BcUPRT*) was expressed only in Inf plants at 9 hpi; *B. cinerea* (BCR16) spore germination can be seen between 6 hpi and 9 hpi at microscope in *B. cinerea* infected tomato leaves (Figure 3.3). Finally, BC1T_13367, a *B. cinerea* polygalacturonase (PGs) precursor gene, expression was earlier and stronger in Inf than Chito+Inf at 9 and 12 hpi (Figure 3.23b).


(a)
Treatment water_infected , Time 6.0 hours
Treatment chitosan_infected , Time 6.0 hours
Treatment water_infected , Time 9.0 hours
Treatment chitosan_infected , Time 9.0 hours
Treatment water_infected , Time 12.0 hours
Treatment chitosan_infected , Time 12.0 hours
Expression
5.0
4.0
3.0
2.5
2.0
1.5
1.2
1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0


(b)
uracil phosphoribosyltransferase expression
Ctrl+Inf
Chitosan+Inf
6hpi
9hpi
12hpi
Time after inoculation (hpi)
PGs precursor expression
Ctrl+Inf
Chitosan+Inf
6hpi
9hpi
12hpi
Time after inoculation (hpi)

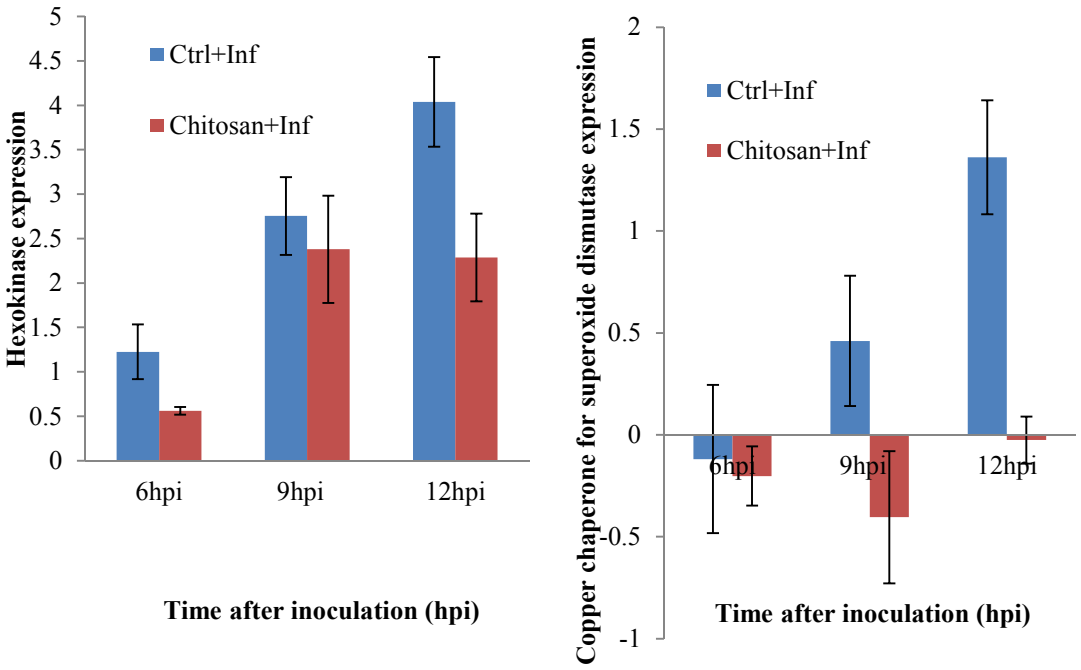


Figure 3.22 *Botrytis cinerea* gene expression profile on Chito+Inf and Inf treatments; (a) Heatmap of *B. cinerea* genes found in microarray analyses from the ANOVA-BH. Hierarchical cluster analyses in GeneSpring software. The colour scale represents gene up-regulation (red), no expression (yellow) and down-regulation (blue). Every column represents a different treatment at the 3 time points (6, 9 and 12 hpi); (b) Normalized gene expression. The data shown are the means of four biological replicates ± standard error of the mean (SEM). Ctrl+Inf, water-treated and *B. cinerea*-infected (Inf) plants; chitosan+Inf, chitosan-treated and infected plants (Chito+Inf). Test Type: Parametric test, differentially expressed genes were defined by Fold Difference: 2 and a P-value < 0.05.

3.3.4 Phytohormone role on chitosan and MeJA-priming for resistance tomato against *Botrytis cinerea*: HPLC/MS analysis

S. lycopersicum transcriptome analysis revealed jasmonic acid (JA) as a key hormone in chitosan-priming tomato against *B. cinerea*, together with a possible cross-talk among other plant hormones such as abscisic acid, salicylic acid and ethylene (Figures 3.13, 3.15 and 3.22). Furthermore, transcriptomic data shows chitosan-priming effect on JA-marker regulatory genes (*SlMYC2* and *SlJAZ1*) together with, SA, ABA and ET-dependent genes, such as *SlNPR1-1*, *SlABAPYL4* and *SlAP2-ERF* respectively (Figure 3.22). Thus, these hormones seem to play key roles in chitosan-priming for tomato resistance against *B. cinerea* during the early stages of the infection.

3.3.4.1 MeJA/chitosan-induced resistance phenotype of the phytohormone analysis

To determine the role of main phytohormone pathways JA, SA and ABA in chitosan-IR in tomato-*Botrytis cinerea* interaction a quantitative determination of the abundance of three acidic plant hormones from a single crude extract directly by LC/MS/MS was performed following a novel methodology approach (Forcat et al. 2008). The method exploits the sensitivity of MS and uses multiple reaction monitoring and isotopically labelled samples to quantify the phytohormones abscisic acid, jasmonic acid and salicylic acid in tomato leaf tissue (Forcat et al. 2008).

Four-week-old tomato plants were treated with two different resistance elicitors, Methyl-jasmonate (MeJA), chitosan and a combination of both elicitors (chitosan + MeJA) in order to look for potential synergies. Five days later, tomato whole leaves were excised and challenged with *B. cinerea* (as described previously). Leaf discs were harvested and freeze dried at three early time points during symptomless stage of the infection (6, 9 and 24 hpi) and evaluated for hormone analysis through HPLC/MS (see M&Ms).

Phytohormones SA/JA/ABA expression was measured in tomato cv. Moneymaker plants infected with *B. cinerea* under four different conditions (and their mock controls). (i) ddH_2O-treated + mock inoculated (Control); (ii) ddH_2O-treated + infected (Inf); (iii) MeJA-treated + mock inoculated (MeJA+mock); (iv) MeJA-treated + infected (MeJA+Inf); (v) chitosan-treated + mock inoculated (Chito+Mock); (vi) chitosan-treated + infected plants (Chito+Inf); (vii) combination (chitosan + MeJA)-treated + mock inoculated (Combo+Mock) and (viii) combination (chitosan + MeJA)-treated + infected plants (Combo+Inf).

Resistance phenotype (infection/pathogenicity assay) analysis revealed that chitosan and MeJA alone and in combination were able to induced resistance and protect tomato against *B. cinerea*, being able to significantly reduce necrotic lesion expansion at 48, and 72 hours post infection (hpi) (Figure 3.24).

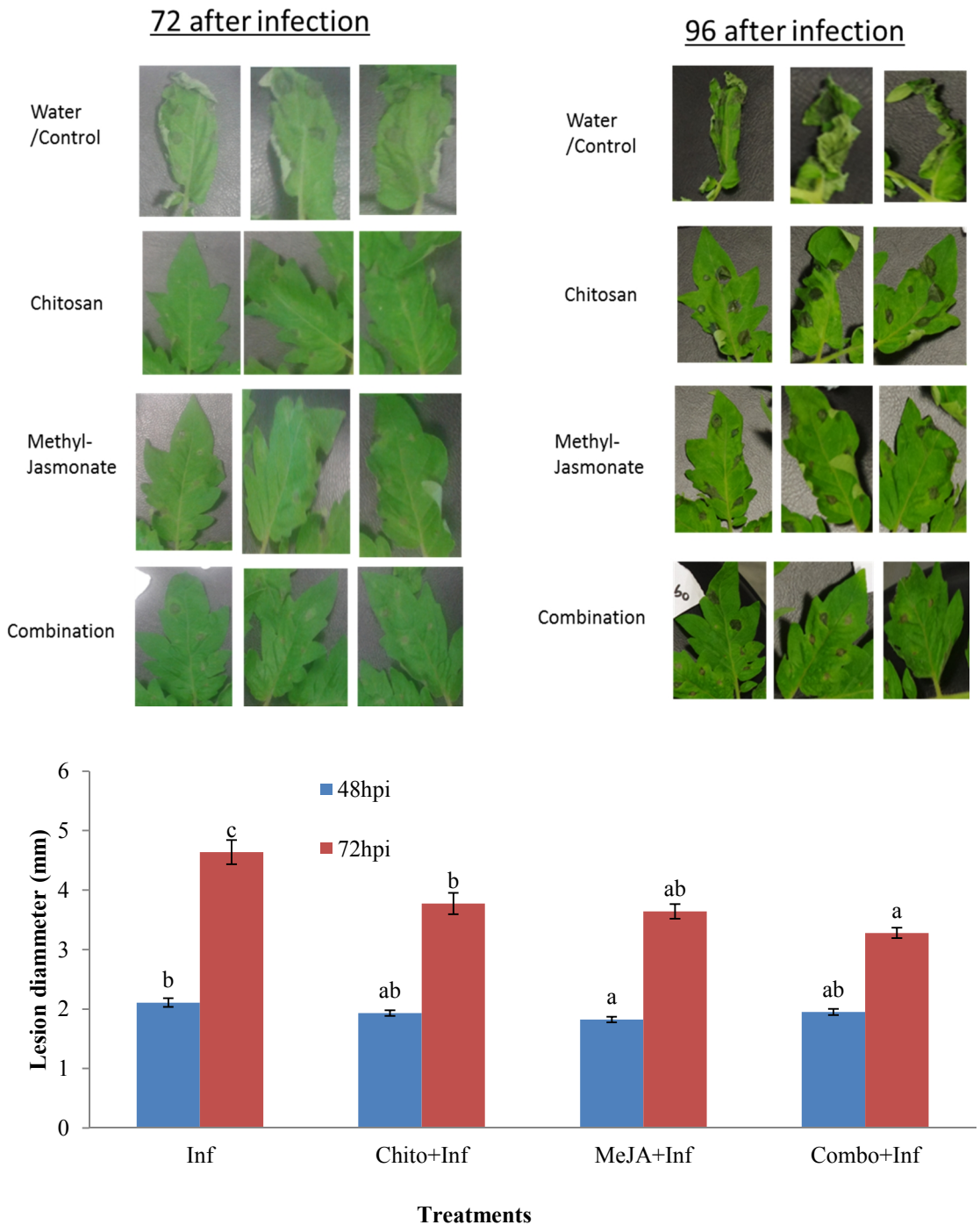


Figure 3.23 Tomato plants treated with chitosan and infected (Chito+Inf), methyl-jasmonate (MeJA)-treated and infected (MeJA+Inf), both elicitors combined and infected (Combo+Inf) and water (non-primed control)-treated and infected (Inf). Four days after treatment, plants were challenged with *Botrytis cinerea* spores. Primed tomatoes showed a stronger protection at 2, 3 and 4 days after infection. Different letters indicate statistically significant differences among treatments (ANOVA followed by Bonferroni Test, $P<0.001$ at 2 dpi and $P=0.005$ at 3 dpi, $\alpha=0.05$).

3.3.4.2 Phytohormone quantification changes in chitosan, MeJA and chitosan+MeJA-treated and *Botrytis cinerea*-infected *Solanum lycopersicum* plants

HPLC/MS hormone analysis revealed jasmonic acid (JA) and its bioactive compound jasmonoyl-isoleucine (JA-Ile) as significantly induced by chitosan and/or MeJA in pathogen presence (Chito+Inf, MeJA+Inf) and absence (MeJA+Mock) at 9 and 24 hpi (Figure 3.25). Treatment with Methyl-jasmonate (MeJA) significantly induced jasmonic acid (JA) without infection (MeJA+Mock) as well as it was able to prime JA hormone levels earlier and stronger during *B. cinerea* infection (MeJA+Inf) in comparison with non-treated + infected plants (Inf) by significantly increasing JA concentration at 9 and 24 hpi (Figure 3.25). In contrast, treatment with chitosan itself (Chito+Mock) and in combination with MeJA (Combo+Mock) supressed MeJA-induced JA levels (MeJA+Mock) at 9 and 24 hpi (Figure 3.25); instead chitosan significantly primed JA (Chito+Inf) later during the infection, at 24 hpi (Figure 3.25), which correlates with the chitosan-induced shift of JA-dependent defences through *SlMYC2* and *SlJAZ1* at 12 hpi seen in the transcriptomic analysis (Figure 3.22). Meanwhile, unlike MeJA and chitosan by themselves, the combination treatment did not induce JA without infection (Combo+Mock) nor primed JA after *B. cinerea* infection (Combo+Inf) (Figure 3.25).

Regarding JA-Ile, significant differences among all treatments were found at 24 hpi (Figure 3.25). In non-infected treatments, chitosan (Chito+Mock) and the combination (Combo+Mock) were able to significantly induce JA-Ile but not MeJa (MeJa+Mock). For infected treatments, JA-Ile was significantly induced in water-treated + infected (Inf) plants at 24 hpi together with MeJA-treated and infected samples (MeJA+Inf); chitosan was also able to significantly induce (prime) JA-Ile after the infection (Chito+Inf) much more strongly than the rest of treatments, which correlates with chitosan-priming JA after the infection (Chito+Inf) at 24 hpi (Figure 3.25). Last, combination treatment did not induce significantly JA-Ile at any time point after the infection (Combo+Inf) linking with no significant induction seen of JA.

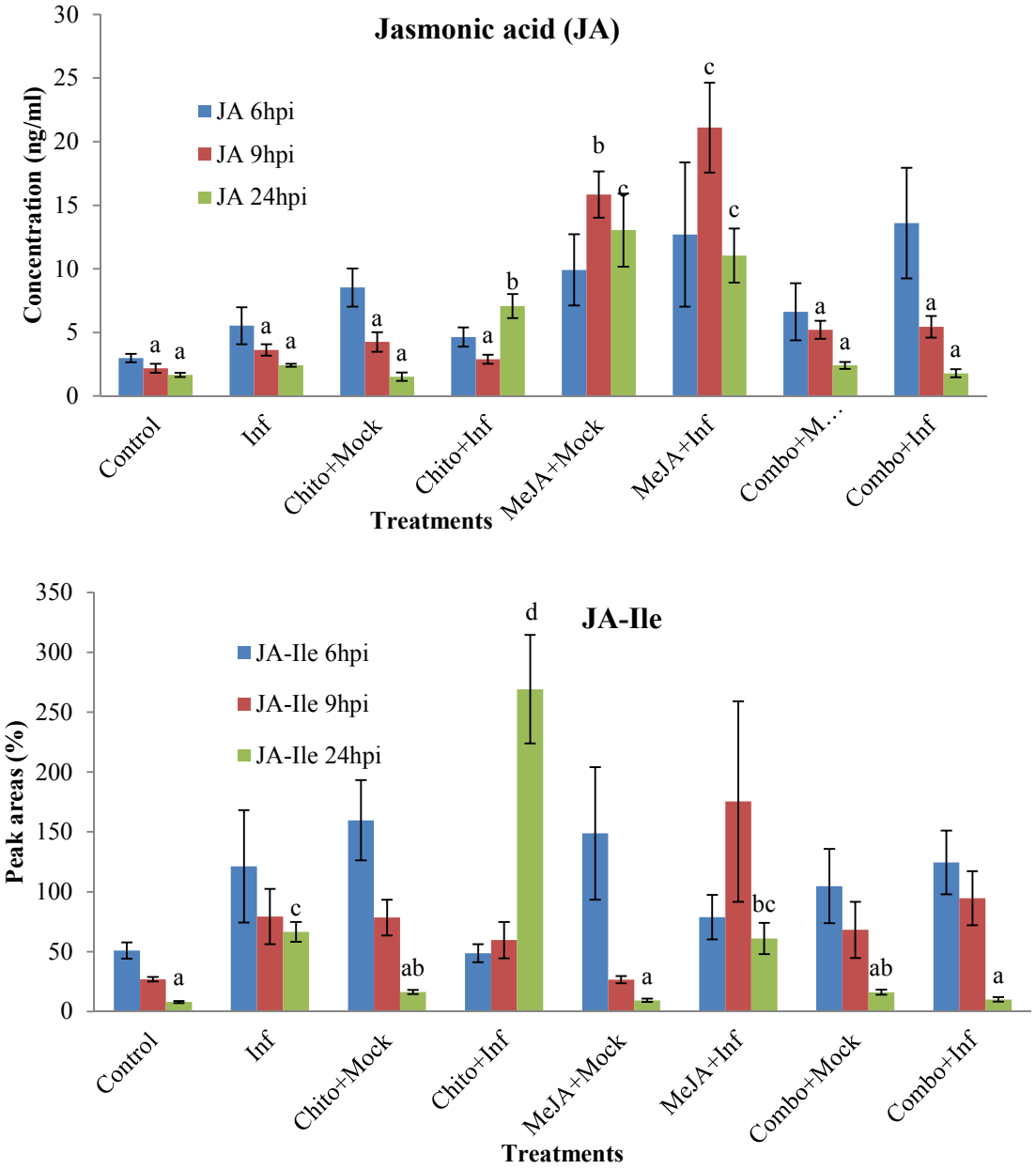


Figure 3.24 Quantification of endogenous JA (ng/g dry weight) and bioactive hormone JA-Ile (% peak area) levels in tomato cv. Moneymaker plants following infection with *B. cinerea* infection for 6, 9 and 24 hpi. (i) WM, Water-treated and non-infected (Control/mock); (ii) Inf, water-treated + infected, (iii) Chito+Mock; chitosan-treated + non-infected (mock); (iv) Chito+Inf, chitosan-treated + infected; (v) MeJa+Mock, MeJA-treated + non-infected (mock); (vi) MeJA+Inf, MeJA-treated + infected; (vii) Combo+Mock, chitosan+MeJA-treated + non-infected (mock); (viii) Combo+Inf, chitosan+MeJA-treated + infected. Values presented are means ± SEM and different letters mean significant differences among all treatments per time point and they were obtained from an ANOVA and then pairwise Fisher's protected least significant difference (LSD) test ($P<0.001$ at 9 hpi and 24 hpi, $\alpha=0.05$; $P=0.194$ at 6hpi for JA; $P=0.221$ for 6 hpi and 9 hpi and $P<0.001$ at 24 hpi for JA-Ile).

In contrast, abscisic acid and salicylic acid were not significantly induced in any of the treatments (Figure 3.26). It was observed that ABA concentration levels were ten times higher than SA and JA (Figures 3.25 and 3.26). However, it was also noticed that ABA levels were reduced by the pathogen itself (Inf) in comparison with non-treated and non-infected treatment (Control) at 6 hpi (Figure 3.26), which suggests a putative pathogen-caused reduction at 6 hpi.

HPLC/MS analysis also showed that MeJA (MeJA+Inf) was able to, although not significantly, trigger synergistically the 3 hormones ABA, SA and JA at 9 hours after the infection (Figures 3.25 and 3.26).

This suggests that SA and ABA hormones might not play a direct role in chitosan-induced resistance in tomato-*B. cinerea* interaction instead of having an indirect but key role in their cross-talk with jasmonic acid and ethylene through transcriptional regulation (Figure 3.22).

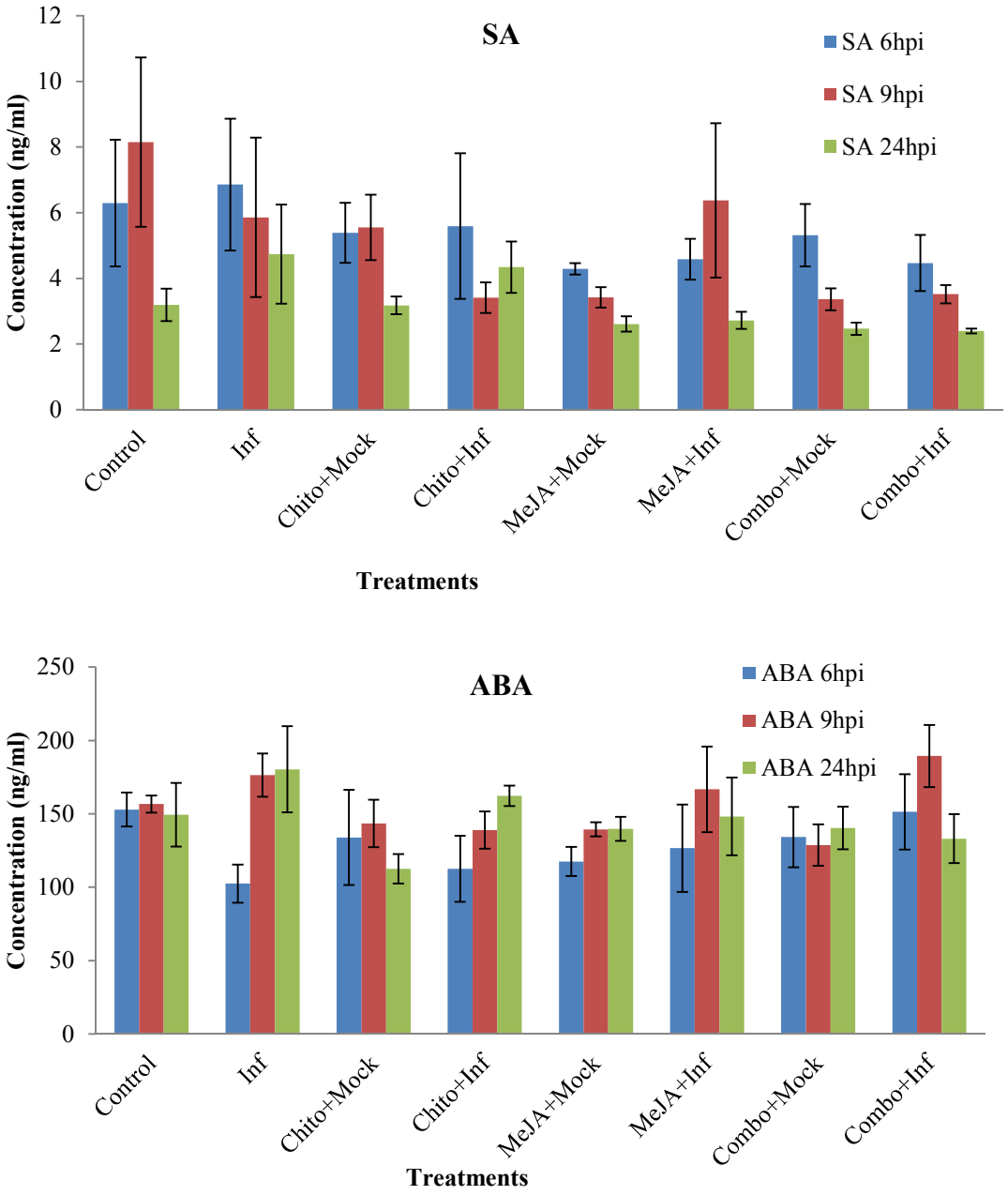


Figure 3.25 Quantification of endogenous SA and ABA (ng/g dry weight) levels in tomato cv. Moneymaker plants following infection with *B. cinerea* infection for 6, 9 and 24 hpi. (i) WM, Water-treated and non-infected (Control/mock); (ii) Inf, water-treated + infected, (iii) Chito+Mock; chitosan-treated + non-infected (mock); (iv) Chito+Inf, chitosan-treated + infected; (v) MeJa+Mock, MeJA-treated + non-infected (mock); (vi) MeJA+Inf, MeJA-treated + infected; (vii) Combo+Mock, chitosan+MeJA-treated + non-infected (mock); (viii) Combo+Inf, chitosan+MeJA-treated + infected. Values presented are means ± SEM and ANOVA was performed to look for significance among treatments (P=0.842, P=0.28, P=0.125 at 6 hpi, 9hpi and 24 hpi respectively for SA; P=0.194 at 6hpi for ABA.

It was observed that the combination treatment highly protected tomato plants as infected leaves had less level of disease than the other treatments (Figure 3.24).

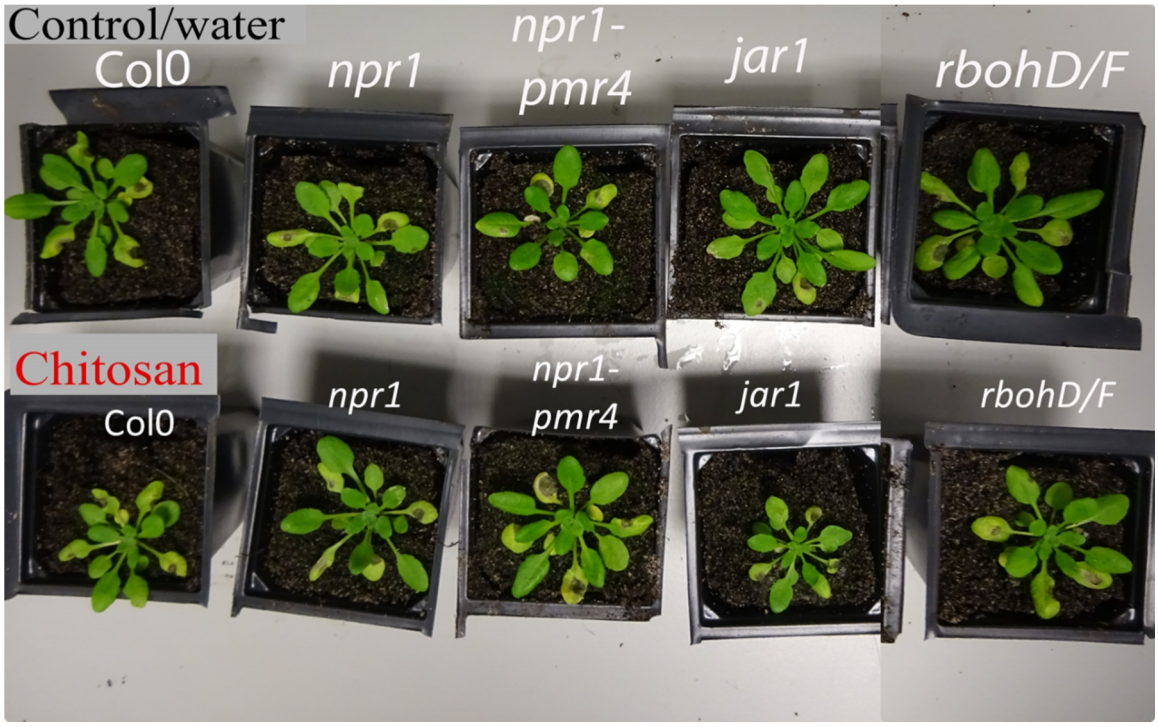
Also, hormone levels were not higher in the combination treated tomatoes in comparison with the single-treated plants (Figures 3.25 and 3.26).

3.3.5 Identification of marker pathways in chitosan-induced resistance in *Arabidopsis thaliana* against *Botrytis cinerea*

Transcriptomic analysis has shown that chitosan can prime key defence pathways in tomato to fight back *B. cinerea* infection, such as cell-wall processes, proteolysis, signalling-receptor and redox processes (Figures 3.13, 3.14, 3.15 and 3.16). Furthermore, stress-related hormonal pathways were primed by chitosan in response to *B. cinerea* infection (Figure 3.22), including auxins, abscisic acid, salicylic acid, ethylene and jasmonate related genes as well as jasmonic acid and JA-Ile themselves (Figure 3.25).

Arabidopsis thaliana is the most well-studied plant system and the numerous genetic studies and resources make it a great candidate to study specific pathways that otherwise would be difficult or time consuming. Chitosan is able to induce resistance in *A. thaliana* Col-0 against *B. cinerea* in a concentration dependent manner (Figure 2.5). Thus, this resistance phenotype in *A. thaliana* wild-type plants together with the wide-range KO lines availability, give us the opportunity to study key hormonal and other defence pathways and their role in chitosan-priming for resistance against this model necrotrophic fungal pathogen. Thus, the following study aims to determine which hormone pathways are involved in chitosan-IR in *A. thaliana* and to investigate possible similarities with chitosan-IR in tomato against *B. cinerea*; Arabidopsis Col-O, *npr1*, *npr1-pmr4*, *jar1* and *rbohD/F* plants were treated with chitosan 0.01% w/v and ddH_2O (control). Four days after treatment, plants were infected with a spore solution of *B. cinerea* by drop inoculation (see M&Ms).

Chitosan (Chito+Inf) significantly induced resistance only in Col-O (WT) against *B. cinerea* compared to water-treated + infected (Inf) plants at 2 dpi (Figure 3.27). Chitosan-induced resistance phenotype did not occur on *npr1*, *npr1-pmr4*, *jar1* and *rbohD/F* KO lines, which suggest a pivotal role of SA, JA, callose and ROS pathways in chitosan-IR in *A .thaliana* against *B .cinerea*.



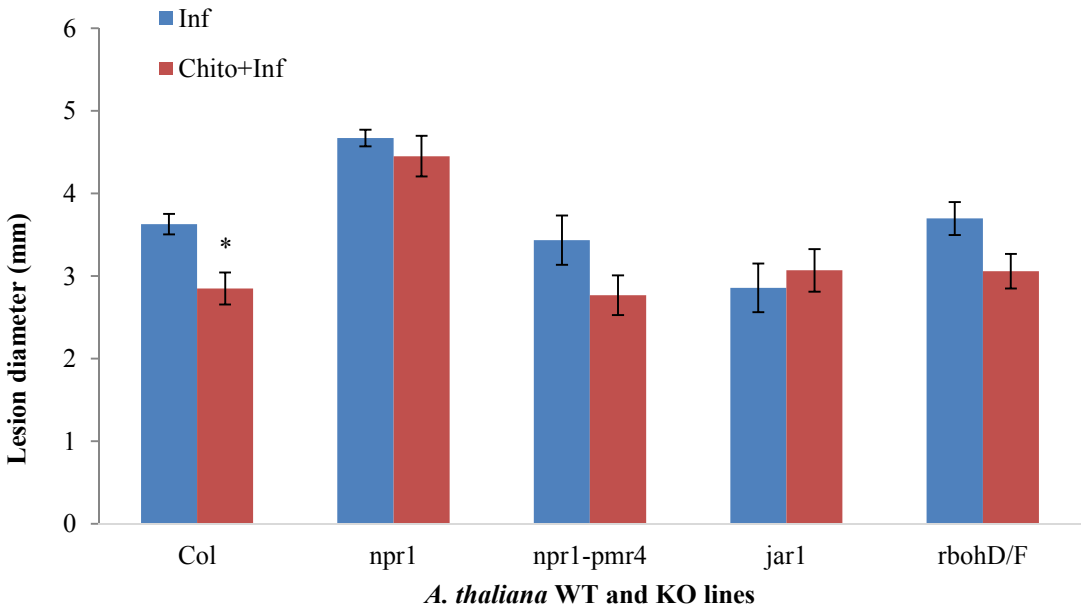


Figure 3.26 Quantification of Chitosan-induced resistance in *A. thaliana* (0.01% w/v) against *B. cinerea* at 2 days post-inoculation (dpi). Values presented are means ± SEM. Asterisk indicate statistically significant differences (SHAPIRO-WILK t-test $P<0.05$ at 2 dpi).

3.4 DISCUSSION

Here, transcriptomic analysis has demonstrated that chitosan is capable of priming and hence fine-tuning *S. lycopersicum* seedlings immune system through a more robust, efficient and rapid defence response against *B. cinerea* infection. Previous analysis on Chapter 2 suggested a 'priming effect' of low-dose chitosan, where low-concentration of chitosan significantly reduced *B. cinerea* lesion expansion in various plants and it was able to induce tomato initial defence mechanisms, such as callose deposition, without having a direct fungicide effect against *B. cinerea*. Chitosan has been widely used, by foliar application in the plant, to control disease development caused by numerous pests and pathogens (El Hadrami et al. 2010). However, few studies have investigated the role of chitosan as a priming agent in agriculture and those studies have mostly focused on the use of chitosan as a seed priming elicitor mainly to improve germination and yield of various crops (Guan et al. 2009; Hameed 2014). Hence, this novel approach aims to investigate the properties of chitosan as a priming foliar treatment in one of the most economically important horticultural crop worldwide, *S. lycopersicum*.

Thus, the main objective of this chapter was to characterise the whole tomato transcriptomic response after *B. cinerea* challenge and to decipher how a well-known MAMP/PAMP (chitosan) can influence (prime) plant transcriptome to fine-tune tomato defences against necrotrophic pathogen attack. In addition to the transcriptome characterisation, as Figure 3.18 shows, candidate differentially-expressed genes (DEGs) that were primed by chitosan (Chito+Inf) at any or various time points during the infection were identified. Their expression was further quantified by an alternative method (qRT-PCR), which had the benefit of validating the microarray data (Figure 3.18). This validation adds value to the experiment and increases the confidence level of the transcriptomic analysis findings, such as pathways and novel candidate genes involved in resistance against *B. cinerea*.

Transcriptomic analysis showed (Figure 3.5) that chitosan (Chito+Inf) can prime *S. lycopersicum* for a faster (earlier gene expression during the infection time points in Chito+Inf vs. Inf) and more robust (higher amount of DEGs in Chito+Inf vs. Inf) defence response against *B. cinerea*, with the significant expression of 1,974 genes whereas non-primed (ddH_2O-treated) and infected (Inf) plants only significantly

expressed 687 genes in chitosan absence during all infection. This type of defence response clearly shows a priming profile as it has been defined before (Ton et al. 2009; Conrath 2011; Mauch-mani et al. 2017). Interestingly, key processes that were induced and enriched by Chito+Inf during the infection belonged to cell-wall-associated receptor kinases, mitogen-activated protein kinases (MAPKs), gluthatione-S-transferases (GST), auxin-related genes, Gibberellin 2-oxidases, peroxidases and Chlorophyll a-b binding proteins, some of which are marker pathways triggered upon MAMP/PAMP recognition in plants (Boudsocq et al. 2010); and transcriptional factors such as APETALA2/Ethylene-responsive element binding protein family, ethylene responsive transcription factors, WRKYs and MYBs. Moreover, it was recently found that calcium-related protein kinases are required in basal and oligogalacturonides (OGs)-induced resistance in *A. thaliana* against *B. cinerea* which directly affect ethylene synthesis (Gravino et al. 2015). This can be observed also during this transcriptomic analysis were signalling receptor-like kinases, calcium-dependent protein kinases and ethylene-dependent processes were highly induced/primed by chitosan (Chito+Inf). Taken together, this indicates a possible cross-talk between mitogen-activated protein kinase (MAPK) cascades and ethylene production primed by chitosan in tomato against *B. cinerea*.

Interestingly, it was observed in gene expression pattern graphs that the amount of down-regulated DEGS was more than three times as great as the up-regulated ones for both Chito+Inf (1,611 down-regulated and 363 DEGs up-regulated) and Inf (366 down-regulated and 321 up-regulated DEGs) treatments (Figures 3.6, 3.7 and 3.8), which indicates that tomato plants might try to repress potential susceptible factors and chitosan primes the plant to increase this repression in order to reduce *B. cinerea* putative host defence manipulation (Temme & Tudzynski 2009; El Oirdi et al. 2011).

To support this theory, the repression of various negative regulators of plant defences (Lund et al. 1998; La Camera et al. 2011) was observed in biotic stress response and metabolic graphs, including ethylene-dependent genes early during the infection (6 hpi) and redox state glutaredoxins (and thioredoxins) during all infection in primed (Chito+Inf) plants whereas non-primed (Inf) plants did not repress them. ROS seems to be required for chitosan-IR in *A. thaliana* since chitosan did not induce resistance in the ROS-deficient double mutant *rbohD/F* (Torres et al. 2002), which indicates that

chitosan might regulate and/or reduce the oxidative stress that is caused by *B. cinerea* infection and it is crucial for the outcome of the host fight against this necrotrophic pathogen (Temme & Tudzynski 2009; Asselbergh et al. 2007). Furthermore, *A. thaliana* glutaredoxin (*ATGRXS13*), repressed by JA, was found to be a susceptibility factor that *B. cinerea* uses to facilitate infection (La Camera et al. 2011). However, in order to test this theory further analysis need to be done (e.g. functional analysis on one or various of the putative susceptible factors and transforming tomato to obtain overexpression or KO lines which would confirm whether these lines are more susceptible/resistance respectively).

Moreover, this chitosan-induced down-regulation of potential susceptible factors was also noticed with nucleotide degradation processes (hydrolases) where Chito+Inf repressed their expression whereas Inf plants did not suppress them (Figure 3.9). The cell-wall heatmap confirmed that Chito+Inf plants were able to strongly repress cell-wall degrading enzymes such as polygalacturonases (Figure 3.21) and pectin methylesterases, which were enriched by Chito+Inf at 12 hpi, which in turn they can facilitate *B. cinerea* growth and degradation of the plant pectin rich cell-wall (Lionetti et al. 2007). Furthermore, transcriptional factor *SlLBD42* was repressed by Inf and its repression was increased in Chito+Inf (Figure 3.18); LATERAL ORGAN BOUNDARIES (LOB) DOMAIN (LBD)-CONTAINING PROTEIN20 (*AtLBD20*) was found to inhibit jasmonic acid (JA)-dependent defences against necrotrophic fungus *Fusarium oxysporum* (Thatcher et al. 2012).

In addition to identifying potential factors associated with susceptibility to *B. cinerea*, this research has uncovered a number of cell-wall related genes that chitosan primes to fight back *B. cinerea* infection. Together with the repression of degradation enzymes (hydrolases) mentioned before, heatmap cluster analysis showed that chitosan (Chito+Inf) induces a faster and stronger expression (priming state) of cell-wall cellulose synthesis genes such as COBRA-like and polymyxin resistance protein (ArnA) than non-primed (Inf) plants (Figure 3.20). Interestingly, chitosan did not induce resistance in the *A. thaliana* SA/callose-deficient mutant *npr1-pmr4* (Nishimura et al. 2003) (Figure 3.27), which indicates that these pathways are required for chitosan-IR in *A. thaliana*. Thus, this suggests a chitosan-induced reinforcement of the cell-wall to decrease fungal penetration in tomato and *A. thaliana*. Chitosan has therefore

demonstrated to prime cell-wall processes in two higher plant systems, which can be crucial to decrease fungal penetration and/or degradation of host cells during initial stages of the infection. This chitosan-primed cell-wall enhancement can be thereby potentially effective in other crops of the *Solanaceae* and *Brassicaceae*.

It is well-known that plant hormones play important roles in the regulation of the defence signalling network upon perception of biotic or abiotic stress (Pieterse et al. 2012; Forcat et al. 2008). Results showed that jasmonic acid seems to be a key hormone in chitosan-plant-*B. cinerea* interaction. JA signalling-deficient mutant line *jar1* (Aranega-Bou et al. 2014; Staswick et al. 2002) did not display a significant induced resistance phenotype (Figure 3.27), which shows how JA pathway seems to be required for chitosan-IR in *A. thaliana* against *B. cinerea*. In order to elucidate the role of key hormonal pathways in chitosan-tomato-*B. cinerea* interaction, significant expression of jasmonic acid (JA), salicylic acid (SA), abscisic acid (ABA) and ethylene (ET)-related genes in the microarray was investigated (Figure 3.22).

Gene expression graphs show how chitosan is able to prime tomato plants and hence fine-tune hormonal cross-talk to combat *B. cinerea* infection. MYC2 is a basic helix-loop-helix (bHLH) domain–containing transcriptional factor (TF) that can act both as an activator and repressor of distinct JA-responsive gene expression in *A. thaliana* (Lorenzo et al. 2004). *SlMYC2* was repressed in primed (Chito+Inf) and non-primed (Inf) plants at 6 and 9 hpi whereas this repression was abolished in Chito+Inf plants at 12 hpi. Jasmonate Zim Domain proteins (JAZ) are negative regulators of JA-induced gene expression (Wasternack & Hause 2013); *SlJAZ1* was showed a similar pattern to *SlMYC2*, being up-regulated in primed and non-primed plants at 6 hpi and 9 hpi whereas Chito+Inf-induced *SlJAZ1* up-regulation started to decrease at 12 hpi. This suggests a possible shift of JA-induced defences at 12 hpi where chitosan no longer represses JA-induced defence expression in favour of other pathways that might be crucial to fight *B. cinerea* in the early stages of the infection and when the pathogen manages to overcome these "relative early defences" chitosan instead primes JA-defence expression.

There is a complex network in the JA-SA cross-talk in response to pathogen attack (Van der Does et al. 2013). JA and SA are generally considered antagonistic pathways (Takahashi et al. 2004), in *A. thaliana*, the defence regulatory protein

NONEXPRESSOR OF PR GENES1 (NPR1) was identified as a key signalling protein in the regulation of SA/JA crosstalk (Van der Does et al. 2013). Thus, NPR1 expression was studied, *SlNPR1-1* was significantly expressed in the ANOVA-BH and it was down-regulated by both non-primed (Inf) and primed (Chito+Inf) tomatoes at 6 hpi but it was only repressed by primed plants at 9 hpi and 12 hpi (Figure 3.22). This suggests that chitosan might reduce *B. cinerea* JA-dependent defence manipulation through induction of NPR1 protein in tomato at later stages of the infection.

The role of ethylene (ET) in the plant immune response to various pathogens is versatile; in tomato it was found to be required for resistance against *B. cinerea* (J. Diaz et al. 2002), however ethylene is also involved in fruit ripening which might ultimately be utilized by *B. cinerea* to induce ripening-related processes and promote susceptibility (Cantu et al. 2009). Interestingly, this transcriptome study showed ethylene as one of the most induced and hence important hormones at all time points of the infection. To investigate ethylene processes further, a biotic stress pathways overview showed that ethylene pathway was down-regulated by Chito+Inf at 6 hpi and up-regulated by Inf plants throughout all the infection (Figure 3.9), this chitosan initial ethylene repression might be in favour of the up-regulation of other pathways (e.g. cell-wall) required to reduce *B. cinerea* initial penetration and degradation of the plant cuticle. In contrast, ethylene and ERF transcription factors were strongly induced at 9 hpi and enriched by Chito+Inf at 6 hpi (Figures 3.10 and 3.13). Furthermore, the AP2-like ethylene-responsive transcription factor *SlAP2*-like (ERF) (Solyc06g075510.2.1) was primed by chitosan during *B. cinerea* infection. Transcription factors belonging to the APETALA2/Ethylene Responsive Factor (AP2/ERF) superfamily are regulatory proteins involved in multiple processes of the plant, including control of secondary metabolism, development and environmental stimuli (Licausi et al. 2013). Some AP2/ERF members are involved in defence responses, such as ethylene response factors (ERFs) *ERF5* and *ERF6*, which act as positive regulators of JA/ET-dependent defences against *B. cinerea* (Moffat et al. 2012). Moreover, in *A. thaliana*, *ORA59*, an AP2/ERF domain transcription factor is required for JA/ET synergistic signalling and subsequent defence induction of JA-dependent gene *PDF1.2* (Pré et al. 2008). Taken together, this suggests that chitosan might promote JA/ET synergistic cross-talk through priming SlAP2-like (ERF) and hence it may fine-tune tomato defence response against fungal

necrotroph *B. cinerea*, as demonstrated previously by (Moffat et al. 2012) in *A. thaliana*.

ABA is an important phytohormone involved in cross-talk with other hormonal pathways. Thus, to further support chitosan-priming phytohormones cross-talk theory, abscisic acid (ABA)-dependent gene expression was investigated. Interestingly, *SlABAPYL4* was primed (repressed) only by Chito+Inf during all infection and by Inf only at 6 hpi, whereas non-primed + infected (Inf) and chitosan-treated + non-infected (Chito+Mock) plants were not able to repress *SlABAPYL4* expression at 9 and 12 hpi. ABA role can be through supressing SA-, ET- and JA/ET-related defences or a JA signalling positive regulator (Windram et al. 2012; Asselbergh et al. 2008), furthermore, it seems that ABA signalling repression, hence the activation of JA/ET-dependent *ERF1* and *ORA59*, enhances *A. thaliana* resistance to *B. cinerea* (Windram et al. 2012), which again demonstrates how chitosan can function as a priming agent by fine-tuning hormonal cross-talk and how by supressing *SlABAPYL*4 can help to activate JA/ET-dependent defences required to fight against *B. cinerea*.

Transcriptomic results on hormone expression in chitosan-primed tomatoes against *B. cinerea* were further verified by phytohormone analysis. HPLC/MS analysis on SA/JA/ABA accumulation in methyl-jasmonate (MeJA) and chitosan-primed tomatoes, revealed jasmonic acid and its bioactive compound jasmonoyl-isoleucine (JA-Ile) as the most important phytohormones against *B. cinerea* in *S. lycopersicum* (Figure 3.24). MeJA exogenous treatment has been found to promote JA biosynthetic and signalling pathways (Li et al. 2012) and has being shown to prime grapes against *B. cinerea* at low concentrations (Wang et al. 2015). As expected, treatment of tomato plants with MeJA, as an inducer of jasmonates (JAs), significantly induced jasmonic acid (JA) without infection (MeJA+Mock) and MeJA was able to highly prime (MeJA+Inf) JA early during the infection (at 9 hpi) in comparison with non-primed and infected plants (Inf), chitosan-treated and non-infected (Chito+Mock) and chitosan-primed (Chito+Inf) plants. In contrast, chitosan priming followed a different pattern, being able to induce JA and the bioactive compound jasmonoyl-isoleucine in a time-dependent manner, later than MeJA, during the infection (24 hpi), which correlates with chitosan-priming activity on JA-defence related signalling genes (*SlMYC2* and *SlJAZ1*) at 12 hpi shown in the transcriptomic analysis. Furthermore, chitosan alone and in combination with

MeJA was able to supress MeJA-induced JA levels. Together, these findings confirm how chitosan primes for 'early-acting' type (PTI-type) of defence responses, revealed in the transcriptome analysis, including, resistance-type genes activation, MAP-kinase signalling cascades, cell-wall modification genes and reactive oxygen species among others, in the beginning of *B. cinerea* infection to shift for 'late-acting' defence pathways (phytohormones JA/ET) from 12 hpi onwards, which correlates with early repression and later activation of JA-dependent due to *SlMYC2* and *SlJAZ1* transcripts seen during transcriptomic analysis.

Unexpectedly, combination treatment (Combo+Mock and Combo+Inf) did not have an additive effect nor did they primed any of the three phytohormones studied. However, the pathogenicity assay showed that the combination of both elicitors induced a stronger resistance phenotype than the separate elicitors by significantly reducing *B. cinerea* necrotic lesions. Previous studies have showed chitosan *in vitro* and *in planta* synergistic effects with silver and bacteria (Yu et al. 2007; Huang et al. 2011). This suggests that low doses of chitosan can be used in combination with other elicitors/biocontrol/chemical agents to protect crops against *B. cinerea* and other pathogens which can potentially lead into synergistic effects and a more efficient and less stressful plant defence, however its molecular mechanisms need to be further investigated.

MapMan custom diagrams showed that lipid metabolism (fatty acid (FA) desaturation) and secondary metabolites such as, phenylpropanoids were induced by chitosan and by *B. cinerea* during the infection (Figures 3.9, 3.10 and 3.12), this might indicate that in the fight against *B. cinerea*, tomato plants trigger an onset of defence mechanisms that are not restrictive to defence-related responses; including secondary metabolism, such as, fatty acid desaturation, phenylpropanoids and glucosinolates which can also be important for the plant/pathogen interaction and have an indirect effect on plant defences (Kachroo et al. 2001; Kliebenstein et al. 2005).

B. cinerea transcriptomic analysis revealed a chitosan-induced repression of *B. cinerea* early expressed genes (Figure 3.23). Furthermore, novel and well-known virulence factors were triggered by the pathogen during the infection (Inf) and repressed by chitosan (Chito+Inf). These include, uracil phosphoribosyltransferase (*BcUPRT*), recently found as a novel virulence factor involved in *B. cinerea* spore germination and

hence in the establishment of the infection (Gonzalez-Rodriguez et al. 2014), moreover UPRT is a key component of the pyrimidine synthesis pathway, which is important for *Candida albicans* resistance to the antifungal drug flucytosine (Hope et al. 2004) and it seems to be involved in *M. tuberculosis* pathogenicity as well (Villela et al. 2013), which makes it a key target for new antifungal/antibacterial strategies. Copper chaperone for superoxide dismutase (*BcCCSOD*) was also repressed in Chito+Inf condition and activated in non-primed and infected (Inf) plants; superoxide dismutase is involved in oxidative-stress during penetration of the plant cuticle (van Kan 2006) and in lesion development on *Phaseolus vulgaris* (Smith et al. 2014c). Hexokinase, a gene required for *B. cinerea* pathogenicity (Rui & Hahn 2007) and polygalacturonase (PG) precursor expression, which are well-known virulence factors that play a role in tissue colonization (Frías et al. 2016; Gonzalez-Rodriguez et al. 2014; Rui & Hahn 2007) were also repressed/reduced in Chito+Inf plants. Interestingly, these virulence factors followed a logic expression during *B. cinerea* infection progress as spore germination and oxidative burst-related genes were displayed early (6 hpi and 9 hpi) and hexokinase and PGs (more PGs were also expressed, data not shown) were triggered from the beginning of the infection till 12 hpi, presumably to promote disease. Taken together, this indicates that chitosan might prime tomato plants to trigger defence pathways (e.g. cell-wall reinforcement, callose deposition, receptor kinase-related signalling cascades and secondary metabolism) that may reduce *B. cinerea* initial pathogenicity machinery such as virulence factors involved in pathogen germination *in planta* and/or effector that might manipulate host defences and promote disease.

3.5 CONCLUSION

Results of this transcriptomic study demonstrate the theory that chitosan can function as a priming agent when foliar applied, by priming tomato plants in a concentration-dependent manner. High concentrations of chitosan can strongly elicit defences in the plant and protect it against *B. cinerea* but result in negative effects, including cytotoxicity or cell death (see Chapter 2), whereas low concentrations may prime tomato own defence mechanisms to fight back pathogen challenge with less or minimal costs in plant fitness under high biotic stress conditions. Similar discoveries have been found, showing that methyl-jasmonate (MeJA) and β-aminobutyric acid (BABA), when applied as low dose treatments, were able to prime *A. thaliana* and grape plants

respectively with less fitness costs than higher concentrations (van Hulten et al. 2006; Wang et al. 2015).

Transcriptomic and HPLC/MS hormone analysis have unveiled a chitosan strategy in priming tomato immune system against *B. cinerea* during early and crucial stages of the infection. Thus, it was identified that chitosan-priming for resistance against *B. cinerea* can be divided in five main groups. Firstly, chitosan was able to enhance tomato signalling processes through induction of receptor-like kinase cascades and mitogen-activated protein kinases. Secondly, chitosan was able to prime cell-wall-dependent processes by repressing susceptible factors (hydrolases), reducing oxidative stress (glutaredoxins) and inducing cell-wall reinforcement (lignin synthesis, callose deposition and resistance proteins). Thirdly, there was a clear chitosan-priming effect on phytohormone cross-talk were chitosan fine-tuned hormone-related defence pathways. Fourthly, chitosan had a positive effect on secondary metabolism (phenylpropanoid pathway and lipid/fatty acid metabolism), which are known to have an effect on *B. cinerea* (Kliebenstein et al. 2005). Fifthly, chitosan was able to repress *B. cinerea* well-known and novel virulence factors. These results confirm that chitosan can be used as a priming agent against necrotrophic fungal pathogen *B. cinerea* in an important crop and potentially extrapolate to other plants, which may facilitate new antifungal strategies and help to include chitosan as a complement into crop protection strategies.

4. Chapter 4. *ACRE75* and *ACRE180*: Positive regulators in plant defence priming for resistance against *Botrytis cinerea*

4.1 INTRODUCTION

4.1.1 Avr9/Cf-9 rapidly elicited (ACRE) genes role in plant defences

The plant immune system is a complex phenomenon that can counteract numerous microbes and would-be pathogen infections. However, plants depend on their innate immunity as they lack of somatic adaptive defences (Jones & Dangl 2006). One the plant's main and earlier defence responses relies on their sensitivity to detect PAMPS/MAMPS/DAMPs through their cell PRRs and subsequent rapid deploy of PTI and/or ETI. In order to promote infection, plant pathogens have avirulence (*Avr*) genes that encode effector proteins that are able to supress PTI (Stergiopoulos & de Wit 2009; McLellan et al. 2013). *Cladosporium fulvum*, the fungal biotrophic pathogen causative agent of leaf mold disease in tomato, possesses four *Avr* genes that encode the effector proteins Avr2, Avr4, Avr4E, and Avr9 (Stergiopoulos & de Wit 2009), which are recognized by tomato Cf (*C. fulvum*)-2, Cf-4, Cf-4E, and Cf-9 R genes, respectively (Stergiopoulos & de Wit 2009; de Wit et al. 1997; Thomma et al. 2005). Tomato cultivars expressing *Cf-9* are resistant to *C. fulvum* strains that express the avirulence protein *Avr9* (Durrant et al. 2000).

Avr9/Cf-9 rapidly elicited (ACRE) genes are usually expressed as part of the defence response of tobacco and tomato to *C. fulvum* and other stress responses (Durrant et al. 2000), such as elicitors (González-Lamothe et al. 2006), wounding (Rowland et al. 2005; W E Durrant et al. 2000), Avr proteins (Durrant et al. 2000) and pathogen/microbe-associated molecular patterns (PAMPS/MAMPs) (Sonnewald et al. 2012; McLellan et al. 2013). Many ACRE gene functions have been deciphered in previous studies (Durrant et al. 2000; González-Lamothe et al. 2006; Rowland et al. 2005), and they usually encode components of signalling cascades, including transcription factors, protein kinases, and ubiquitination pathway-related proteins, such as F-box and U-box proteins (González-Lamothe et al. 2006). Some ACRE genes have been associated with PAMP-triggered immunity (PTI) responses, such as tomato and *N.*

benthamiana ACRE31 (Sonnewald et al. 2012; Boevink et al. 2016) while others, such as *ACRE74*, *ACRE276* and *ACRE189* are related to hypersensitive response (HR) (Sadanandom et al. 2012; González-Lamothe et al. 2006) as part of the effector-triggered immunity (ETI) response. Furthermore, low doses of *Avr9*, independently of HR, can delay *B. cinerea* development in tomato and *Sclerotinia sclerotium* in Cf-9 oilseed rape plants (Hennin et al. 2002). As mentioned, many ACRE genes are involved in signalling pathways, such as JA-related signalling, Ring-H2, calcium binding, MAP kinase (MAPK)-cascades and ethylene synthesis (Figure 4.1), thus they may play pivotal roles in the initial development of the defence response (Rowland et al., 2005). However, there are ACRE genes roles in plant-pathogen interaction that are still poorly studied, including *ACRE65*, *ACRE180*, *ACRE75*, *ACRE194* and *ACRE169* and their molecular mechanisms remain unknown (Durrant et al. 2000). Thus, ACRE gene roles in plant resistance against necrotrophic pathogens need to be further investigated.

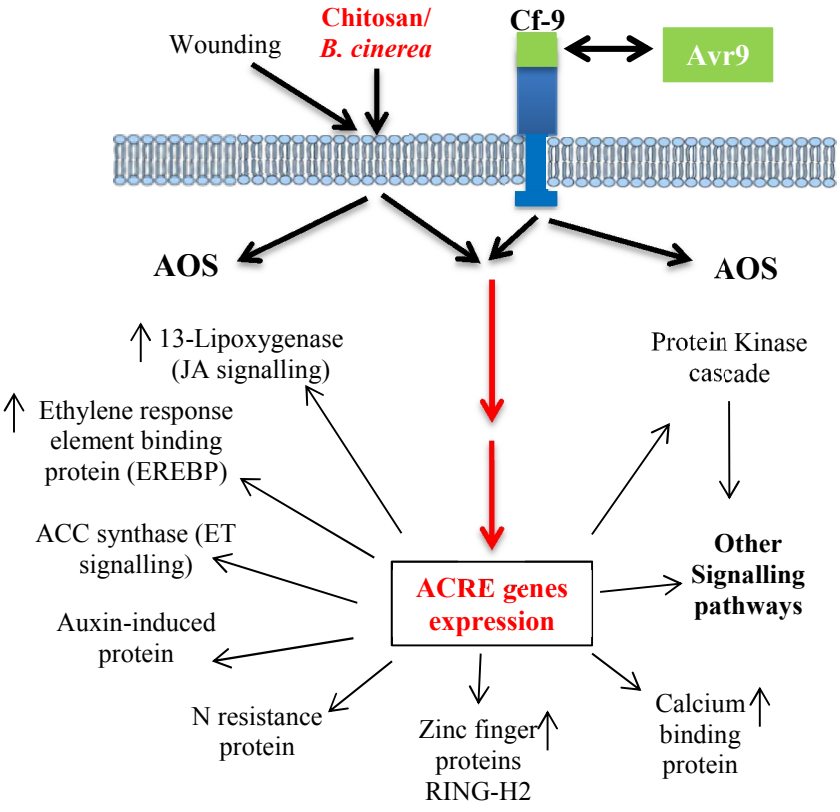


Figure 4.1 Simplified model for the role of Avr9/Cf-9 rapidly elicited (ACRE) genes in plant stress responses (adapted from (Durrant et al. 2000). Upward pointing arrows indicate elevated protein levels. The double-headed arrow indicates a possible interaction between the Cf-9 and Avr9 proteins.

In Chapter 3, transcriptomic analysis revealed many signalling pathways and their related genes, including transcriptional factors (TFs), involved in chitosan-priming for tomato resistance against *B. cinerea*. However, many of these genes have been well-characterised previously as marker genes in the plant immune responses against pathogen attack (Dombrecht et al. 2007; Lackman et al. 2011; Aleman et al. 2016; Wasternack & Hause 2013; Licausi et al. 2013; Cao et al. 1997), such as hormone-dependent *SlMYC2*, *SlJAZ1*, *SlNPR1*, *SlABAPYL4*, *SlAP2-like* (ERF) or cell-wall dependent degradation enzymes/hydrolases (Chapter 3). In contrast, as mentioned before, gene ontology (GO) analysis of large data sets, such as transcriptomics/genomics, can filter uncharacterised genes with unknown functions that might be crucial for plant defence response against a specific pathogen. A transcriptomic overview of biotic stress response pathways analysis, with MapMan, revealed many uncharacterised differential expressed genes (DEGs) triggered by Chito+Inf (Figures 3.9, 3.10 and 3.12) including Avr9/Cf-9 rapidly elicited (ACRE) genes. The main aim of this Chapter is to investigate and characterise the role of two uncharacterised ACRE genes (*ACRE75* and *ACRE180*), identified in the transcriptomic analysis, in chitosan-priming for resistance in tomato and *N. benthamiana*; and the characterisation of *NbACRE31*, *NbPTI5*, *NbWRKY7*, *NbWRKY8* expression, PTI marker genes, and hence important for the initial plant defence responses against *B. cinerea* infection. *NbACRE31*, *NbPTI5*, *NbWRKY7* and *NbWRKY8* have been previously studied as defence-related genes in tomato, *N. benthamiana* and pepper immune responses against *Xanthomonas campestris*, *Phytophthora infestans, Colletotrichum orbiculare* and *B. cinerea* (Sonnewald et al. 2012; Boevink et al. 2016; Ishihama et al. 2011; Adachi et al. 2016).

Subcellular localization analysis can help to decipher gene functions, as it is well-known the importance of cell organelles in plant defences and in the establishment of the plant cell immune dynamics, such as transcriptional factors or MAP kinases for translocation from the cell transmembrane to the nucleus (Motion et al. 2015; Adachi et al. 2015). Furthermore, it has been reported that priming agents such as the non-protein amino acid β-aminobutyric acid (BABA) facilitate organelle translocation within the cell (Luna, van Hulten, et al. 2014). Therefore, Chapter 4 will help to determine the molecular functions of both *ACRE75* and *ACRE180* genes and their implication in crop protection.

4.2 MATERIALS AND METHODS

4.2.1 Identification of SlACRE genes and bioinformatics analysis

Tomato ACRE genes were found within the 2-way Analysis of Variance (ANOVA) (Benjamini-Hochberg corrected) using GeneSpring software (v. 7.3; Agilent Genomics). Solyc11g010250.1.1 (*SlACRE75*) and Solyc08g016150.1.1 (*SlACRE180*) functional description (Sol Genomics Network) was Avr9/Cf-9 rapidly elicited protein 75 and Avr9/Cf-9 rapidly elicited protein 180 respectively.

4.2.2 Identification of SlACRE75/SlACRE180 orthologues in Nicotiana benthamiana

N. benthamiana orthologues were detected (Sol Genomics Network) with reciprocal best BLAST hits (RBBH) for *SlACRE75* and *SlACRE180* CDS and protein, termed *NbACRE75* and *NbACRE180*, respectively, which are similar to both *SlACRE75* and *SlACRE180* from tomato. (i) For *SlACRE75*, Best CDS Hit against *N. benthamiana* genome was Niben101Scf03108g12002.1 sharing a 92.73% identity (Id) and Best Protein Hit was Niben101Scf03108g12002.1 with 77.46% of identity; (ii) For *SlACRE180*, Best and only Protein Hit against *N. benthamiana* genome was Niben101Scf12017g01005.1, with 49.51% of identity.

4.2.3 qRT-PCR analysis of *Nicotiana benthamiana* defence gene expression

4.2.3.1 Generation and treatment of plant material

N. benthamiana (wild-type) seeds were placed into propagators for germination and once germinated were grown in a glasshouse under standard conditions (16h-8h/ day-night cycle; 26° C/22° C) before use (see Chapter 2, M&Ms).

Four-week-old plants were foliar sprayed 4 days prior fungal infection with (i) ddH_2O solution + Tween20 0.01% (adjuvant/surfactant) and (ii) chitosan (ChitoPlant) 0.01% w/v (priming concentration, see Chapters 2 and 3) + Tween20 0.01%. Four days after treatment, 1-2 leaves per whole plant (biological replicate) were excised and subsequently infected, and mock inoculated (ddH_2O) as a non-infected control, with a spore solution of *B. cinerea* (2×10^4 spores/ mL) by drop inoculation as described in the

modified Lancaster protocol/pathogenicity assay (see Chapter 2). Three whole plants were used per treatment. Sample collection was done by harvesting leaf discs with a cork borer surrounding infection site at asymptomatic stages 6, 9 and 24 hour post infection (hpi). Samples were then frozen in liquid nitrogen and subsequently stored in 2 mL tubes at -80 ºC until RNA extraction.

4.2.3.2 RNA extraction, cDNA synthesis and quantitative RT-PCR

RNA extraction was performed with RNeasy Plant MiniKit (Qiagen), Dnased with TurboDnase (ThermoFisher) and complementary DNA (cDNA) was synthesized from 2.5 µg of total RNA using Superscript III reverse transcriptase (Invitrogen) and random hexamer/Oligo DT primers. qRT-PCR were performed with specific *N. benthamiana* primers (Sigma-Aldrich) of *NbACRE75*, *NbACRE180*, *NbACRE31*, *NbPTI5*, *NbWRKY7*, *NbWRKY8*, *NbActin* (reference control gene) and *NbEF1- α* (reference control gene) coding sequences.

NbACRE75, *NbACRE180*, *NbActin* and *NbEF1-α* primers (Appendix) and probes were designed using Universal Probe Library (UPL) assay design center (Roche Diagnostics Ltd.) and qRT-PCR was performed using FastStart Universal Probe Master Mix (Roche); *NbACRE31*, *NbPTI5*, *NbWRKY7*, *NbWRKY8* and *NbEF1-α* primers (Appendix) were designed using PRIMER3plus software and qRT-PCR was performed using SYBR Green Master Mix (Qiagen). Amplification and detection of specific products were performed according to the manufacturer instructions, with the following cycle profile for the UPL primers: denaturation step at 95° C for 10 min; 40 cycles of 95° C for 15 sec, annealing and extension at 60° C for 1 min; and the following cycle for the PRIMER3/SYBR Green primers: denaturation step at 95° C for 15 min; 40 cycles of 95° C for 15 sec, annealing and extension at 60° C for 1 min, and final elongation step at 72° C for 30 sec. Each qPCR reaction contained at least two non-template controls.

All reactions were run in technical triplicates for each biological replicate and the mean values were used for quantification. The relative quantification of target genes was determined using the Pfaffl method (Pfaffl 2001). Gene expression data was relative to the ddH_2O-treated (control) treatment and data represent means of three biological replicates for the qRT-PCR ± standard error of the mean (SEM). Real-time amplification reactions were performed using FastStart Universal Probe Master (Rox)

or SYBR Green detection chemistry (melting curve analysis was performed at the end of each run to ensure that unique products were formed). Quantitative RT-PCRs were performed using a StepOne Real Time PCR System (Applied Biosystems).

4.2.4 Generation of transient overexpression constructs

Gateway® cloning was used to design and produce overexpression constructs for the gene candidates for a N-terminal GFP:ACRE fusion protein per insert. Concisely, flanking the (i) *SlACRE75*, (ii) *SlACRE180*, (iii) *NbACRE180* and (iv) *NbACRE75* coding sequences (CDS) (Sol Genomics Network), 2 nucleotides were added (GC at the beginning of the sequence and GA to create the TGA stop codon at the end) for the stop codon and make it N-terminal fusion compatible. pUC57 (Appendix) plasmids (entry clones) containing *SlACRE75*, *SlACRE180*, *NbACRE75* and *NbACRE180* coding sequences were chemically synthesized by GenScript with gene-specific primers modified to contain the Gateway (Invitrogen) attL1 and attL2 recombination sites and designed with a custom Python script (Dr. Gaetan Thilliez, Quadram Institute, UK).

For *SlACRE75*, *SlACRE180, NbACRE75* and *NbACRE180*, cDNAs from pUC57 entry vector were transformed into *Escherichia coli* strain DH10B by electroporation at a standard voltage for bacteria (1.8 kV with 0.1 cm gap cuvettes) (Bio-rad MicroPulser™ Electroporator with the MicroPulser™ Electroporation Cuvettes, 0.1 cm gap). Transformed cells were spread on LB-plates, each containing 100 µg/ mL ampicillin, 20 µg/ mL X-gal, and 0.1 mM IPTG and incubated at 37° C overnight. The inserts were confirmed by colony PCR with m13/m13rev primers (Appendix) and by sequencing before being introduced into *Agrobacterium tumefaciens*. After confirmation by sequencing and making the glycerol stocks, plasmids were extracted (QIAprep Spin Miniprep Kit) and transferred by a recombinant LR reaction of Gateway cloning (Clonase II enzyme mix Kit, Thermo Fisher) that is catalysed by the enzyme mixture LR clonase II into pB7WGF2 (Karimi et al. 2002). GFP fusions were made by recombining the entry clones with pB7WGF2 (Karimi et al. 2002) (Appendix), placing the cDNA in-frame with GFP on the N-terminus, under the control of the 35S promoter. The constructs obtained were confirmed by sequencing (sequences were aligned to confirm resemblance with the original CDS, BioEdit sequenced alignment editor). The constructs where then used for the transformation of *A. tumefaciens*, strain GV3103 by electroporation and selected on LB-agar plates containing gentamicin (25 µg/ mL),

rifampicin (50 µg/ mL) and spectinomycin (100 µg/ mL). Glycerol stocks of the GV3101 + pB7WGF2 + 4 inserts/empty vector were made and frozen at -80° C via liquid nitrogen.

4.2.5 Agroinfiltration and statistical analysis of pathogenicity assay

Nicotiana benthamiana (wild-type) plants were generated (see 4.2.3.1). *A. tumefaciens* GV3101 carrying plasmids with transient expression constructs, (i) pB7WGF2:35S:GFP:SlACRE75; (ii) pB7WGF2:35S:GFP:SlACRE180; (iii) pB7WGF2:35S:GFP:NbACRE180; (iv) pB7WGF2:35S:GFP:NbACRE75 and (v) pB7WGF2:35S:GFP (empty vector), were grown in YEP medium (50 µg/ mL rifampicin, 100 µg/ mL spectinomycin, and 25 µg/ mL gentamicin) for 24 h with continuous shaking at 28° C. Overnight cultures were collected by centrifugation, resuspended in Agromix/infiltration buffer (10 mM $MgCl_2$: 10 mM MES) and 200 µM acetosyringone (pH 5.7) and diluted to a final volume of 20 mL at OD_{600} of 0.1.

Cultures containing infiltration buffer and overexpression constructs (OD_{600} of 0.1) were agro-infiltrated into leaves (into the abaxial leaf surface) of 4-week-old *N. benthamiana* plants using 1 ml needleless syringes. One day after agroinfiltration, 1-2 leaves per plant were excised for infection/pathogenicity assays (as described in Chapter 2, M&Ms), subsequently challenged with *B. cinerea* spore inoculum (2 x 10^4 spores/ mL) by drop inoculation and lesion size measurements were taken at 3 and 4 dpi with an electronic ruler. Briefly, lesion size (mm) measurements were performed at 4 days post-infection (dpi) on six independent plants and 3 leaves/ construct to look for a resistance phenotype. To look for significance among treatments/constructs data were subjected to analysis of variance (ANOVA) followed by Tukey's Post-hoc at 4 dpi, with 'construct' as a single treatment factor with 5 and 7 levels for experiment 1 (Rep1) and 2 (Rep2) respectively: GFP-empty vector (EV) (control treatment); 'GFP:SlACRE75; 'GFP:SlACRE180'; 'GFP:NbACRE180; and 'GFP:NbACRE75'; 'GFP:Combo1-2'; 'GFP:Combo3-3'. Six plants with 3 leaves per plant were used/infected per overexpression construct. In order to decrease variation within plants, every construct was agroinfiltrated on the right side of the leaf whilst *A. tumefaciens* GV3101 containing pB7WGF2:35S:GFP (empty vector) was infiltrated on the left side of almost every leaf (until all 20 mL used) as a non-protein negative control. The experiment was repeated twice (Rep1 and 2). During the second transient overexpression assay, 2

coexpression treatments were added (vi) pB7WGF2:35S:GFP:SlACRE75 + pB7WGF2:35S:GFP:SlACRE180 and; (vii) pB7WGF2:35S:GFP:NbACRE75 + pB7WGF2:35S:GFP:NbACRE180.

4.2.6 Western blot analysis

Two days after overexpression constructs agro-infiltration (at OD_{600} of 0.5) in *N. benthamiana* leaves, two leaf discs per construct were excised and placed into 2 mL Eppendorf tubes, frozen in liquid N_2 and stored at -80° C.

Leaf discs were ground and added to 200 µL 2 X SDS loading buffer (100 mM Tris–HCl, pH 6.8, 4% SDS, 0.2 % bromophenol blue, 20 % glycerol and 200 mM DDT). The samples were boiled for 10 min at 95° C and subsequently centrifuged at 10,400 x g for 10 min at 4 °C. Supernatant was transferred to a new tube. Gels were assembled into the gel tank and the inner reservoir filled with 200 mL 1XMOPS running buffer containing 500 µL antioxidant and outer reservoir with 1x MOPS running buffer.

Proteins in 20 µL of the supernatant were separated on a 12 % SDS-PAGE gel and transferred onto a nitrocellulose membrane membrane by semi-dry transfer. Gels were blotted onto a nitrocellulose membrane (GE Healthcare Life Science, Amersham Protran Premium 0.45 NC 200 mm x 4 m) for 1.5 h at 30V and stained with Ponceau solution to show loading and transfer. Membranes were blocked within 4% milk solution in 1 X PBST (phosphate buffered saline (137 mM NaCl, 12 mM Phosphate, 2.7 mM KCl, pH 7.4) with Tween20 0.2% (vol/vol)) before addition of the primary antibody. Detection of GFP was performed using a polyclonal rabbit anti-GFP antibody (1:4,000 dilution). The membrane was washed with 1 X PBST (0.2% Tween 20) before addition of the secondary anti-mouse antibody (IG HRP 1:10,000) according to the manufacturer's instructions. Equal loading of proteins in the lanes and transfer efficiency were ensured by visual assessment after Ponceau staining of membranes prior to immunodetection. ECL development kit (Amersham) detection was used according to the manufacturer's instructions and the film was developed using an X-ray developer machine. The original gel images and expected protein sizes are shown in Appendix.

4.2.7 Subcellular localization of *SlACRE75*, *SlACRE180*, *NbACRE75* and *NbACRE180*

For the analysis of the subcellular localization, *A. tumefaciens* GV3101 carrying plasmids with transient expression constructs, (i) pB7WGF2:35S:GFP:SlACRE75; (ii) pB7WGF2:35S:GFP:SlACRE180; (iii) pB7WGF2:35S:GFP:NbACRE180; (iv) pB7WGF2:35S:GFP:NbACRE75 and (v) pB7WGF2:35S:GFP (empty vector), were grown in YEP medium (see 4.2.5). Cultures containing infiltration buffer and overexpression constructs (OD_{600} of 0.1) were co-infiltrated with pFlub vector (RFP-peroxisome tagged marker) into leaves (into the abaxial leaf surface) of 4-week-old *N. benthamiana* CB157 (nucleus mRFP marker) and CB172 (ER mRFP marker) reporter lines using 1 ml needleless syringes. Two days after agroinfiltration, agoinfiltrated leaves were excised and prepared for confocal microscopy. GFP and mRFP fluorescence was examined under Nikon A1R confocal microscope with the following water-dipping objective: Nikon X 40/ 1.0W. GFP was excited with 488 nm from an argon laser and its emissions were detected between 500 and 530 nm. mRFP was excited with 561 nm from a diode laser, and its emissions were collected between 600 and 630 nm.

4.2.8 Generation of *Arabidopsis thaliana* stable overexpression transgenic lines

Gateway® cloning was used to produce the four overexpression constructs for N-terminal GFP:SlACRE75, GFP:SlACRE75, GFP:SlACRE180, GFP:NbACRE180 and GFP:NbACRE75 fusion proteins (4.2.4). Arabidopsis overexpression plants were transformed using the *A. tumefaciens*–mediated flower dipping method (Clough and Bent, 1998). Selection of *A. thaliana* transformants was performed by multiple spray applications of 120 mg/L glufosinate-ammonium PESTANAL® (BASTA; Sigma; 45520). Selected lines were selected on the basis of monogenic segregation of BASTA resistance in T2 progeny (3R:1S) and tested for resistance against *B. cinerea*. Two independent homozygous overexpression lines were obtained per construct.

4.2.9 Basal-induced resistance in *Arabidopsis thaliana* ACRE overexpression lines

The experiment was conducted with the kind collaboration of Dr. Estrella Luna (Sheffield University, UK). *A. thaliana* Col-0 (wild-type), GFP-SlACRE75, GFP-SlACRE180, GFP-NbACRE180 and GFP-NbACRE75 lines described previously were

mass-seeded on soil (Sheffield compost, Chapter 2), grown in cubicle and cultivated under *A. thaliana* standard growth conditions (10h-day (25ºC) and 14h-night (20ºC) cycle at ~60% relative humidity (RH). Ten-day-old plants were transplanted to another pot with a total of 5 plants per pot. Five-week-old plants were infected with *B. cinerea* by drop inoculating leaves with an inoculum containing 5 x 10^5 spores/ mL (see Chapter 2, M&Ms). Disease was scored with the phenotyping scanner and by measuring lesion diameter with an electronic ruler, at 2, 3 and 6 days post infection.

4.2.10 Statistical analysis of pathogenicity assay in *Arabidopsis thaliana* ACRE overexpression lines

Data analysis was performed using GenStat® 18th Edition (VSN International, Hemel Hempstead, UK). Data were subjected to analysis of variance (ANOVA) with 'construct' as a single treatment factor with 10 levels: Col-0 (wild type treatment); two empty vector lines 'EV 3.1' and 'EV 4.1'; 'SlACRE75 1.1' and 'SlACRE75 2.1'; 'SlACRE180 1.2' and 'SlACRE180 3.1; 'NbACRE180 1.1' and 'NbACRE180 2.1'; and 'NbACRE75 1.1'. The replicate units were plants of which there were 8-16 for each construct. Measurements on four lesions were recorded for each plant. Thus the random effects were modelled as Plant + Plant × Lesion to capture the plant-to-plant and within-plant variation. As part of the ANOVA, specific planned (non-orthogonal) contrasts were included to test for significant differences between the mean for each of the genes (*SlACRE75*, *SlACRE180*, *NbACRE180* and *NbACRE75*) compared to Col-0 (wild-type treatment) plants. For example, to test if the gene *S1ACRE75* was significantly different from the wild-type the contrast compares the mean of the two gene constructs versus the wild-type mean. The mean of the two empty vector lines were also compared to Col-0 as GFP- no insert control treatment. This analysis was performed once for each time point.

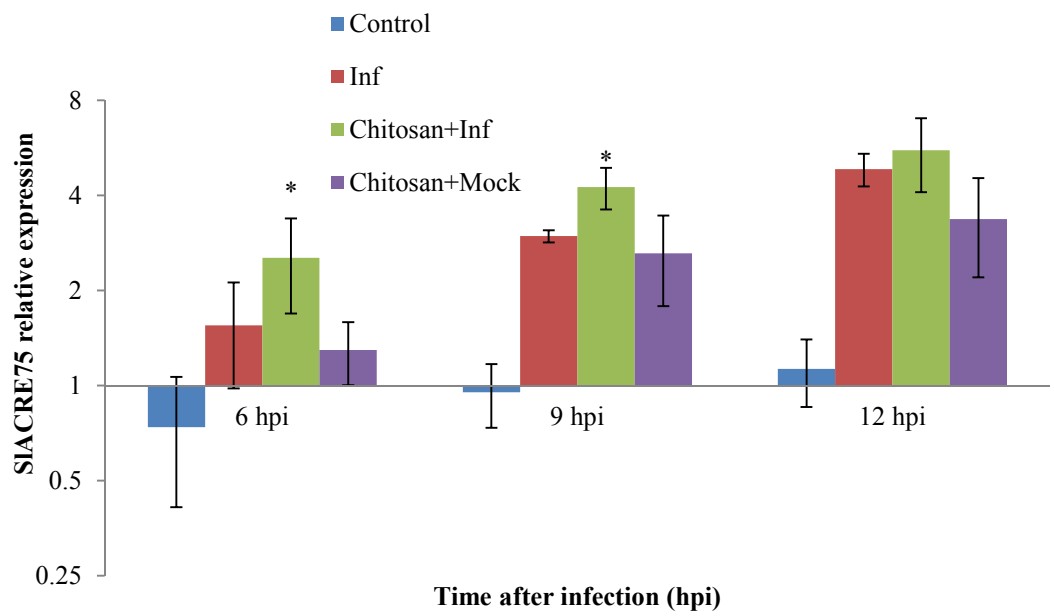
4.3 RESULTS

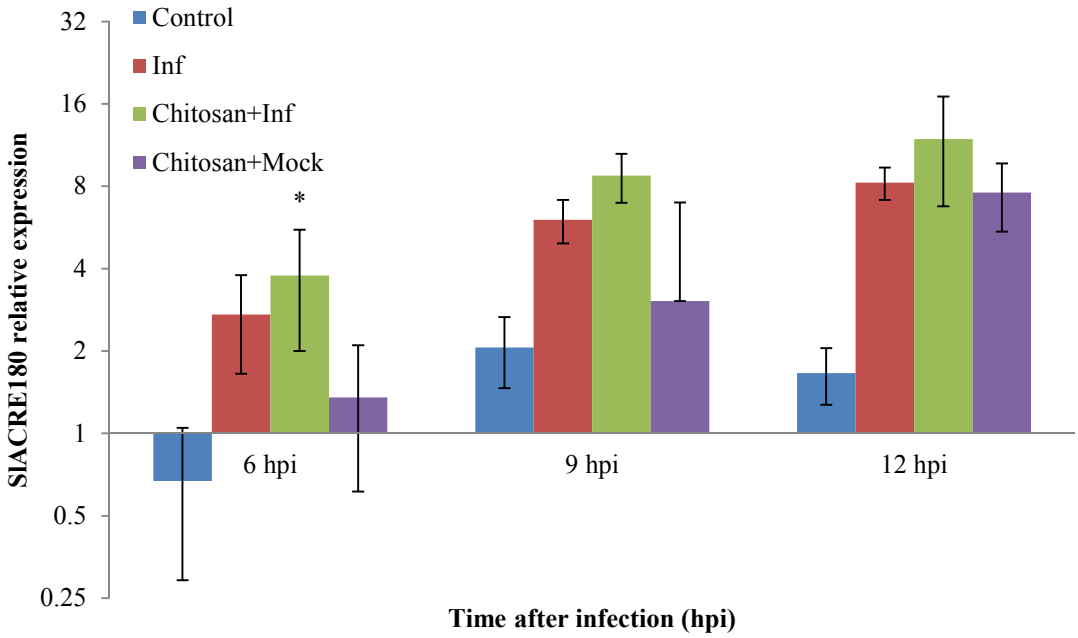
4.3.1 Identification of *SlACRE75* and *SlACRE180* in chitosan-primed and *Botrytis cinerea* infected tomato

As mentioned in Chapter 3, gene ontology (GO) analysis of large data sets, such as transcriptomics/genomics, can filter uncharacterised genes with unknown functions that might be crucial for the plant defence response against a specific pathogen. Overview of biotic stress response pathways analysis with MapMan revealed many uncharacterised differential expressed genes (DEGs) triggered by Chito+Inf (Figures 3.9, 3.10 and 3.12).

Thus, to find novel uncharacterised genes that might be involved in chitosan-priming, DEGs with a priming phenotype (e.g. genes significantly expressed earlier and/or stronger during the infection on Chito+Inf only (Venn Diagrams, Figure 3.5) were investigated. Among all the DEGs spotted from the ANOVA, Avr9/Cf-9 rapidly elicited (ACRE) genes were identified and they were differentially expressed only by Chito+Inf at 6 and/or 9 hpi, including *SlACRE75*, *SlACRE180*, *SlACRE146* and *SlACRE137* (Figure 4.2). The four ACRE genes identified were up-regulated by the pathogen (Inf). By chitosan without infection (Chito+Mock) and their expression was in most cases enhanced by both the priming agent and *B. cinerea* (Chito+Inf) at all three time points after the infection (hpi) (Figure 4.2). *SlACRE75* and *SlACRE180* were significantly up-regulated by Chito+Inf early during the infection, at 6 hpi. At 9 hpi, only *SlACRE75* and *SlACRE146* were significantly expressed by Chito+Inf whereas chitosan did not significantly prime (Chito+Inf) *SlACRE137* during the infection (Figure 4.2). Thus, *SlACRE75* was highly primed earlier and longer during the infection whereas the other ACRE genes were either primed at only one time point post-infection or none.

Moreover, qRT-PCR analysis confirmed high expression levels of both *SlACRE75* and *SlACRE180* as well as a stronger priming effect of *SlACRE180* (Figure 4.3).


Control
Inf
Chitosan+Inf
Chitosan+Mock
8
4
2
1
0.5
0.25
SlACRE75 relative expression
*
*
6 hpi
9 hpi
12 hpi
Time after infection (hpi)


Control
Inf
Chitosan+Inf
Chitosan+Mock
32
16
8
4
2
1
0.5
0.25
SlACRE180 relative expression
*
6 hpi
9 hpi
12 hpi
Time after infection (hpi)

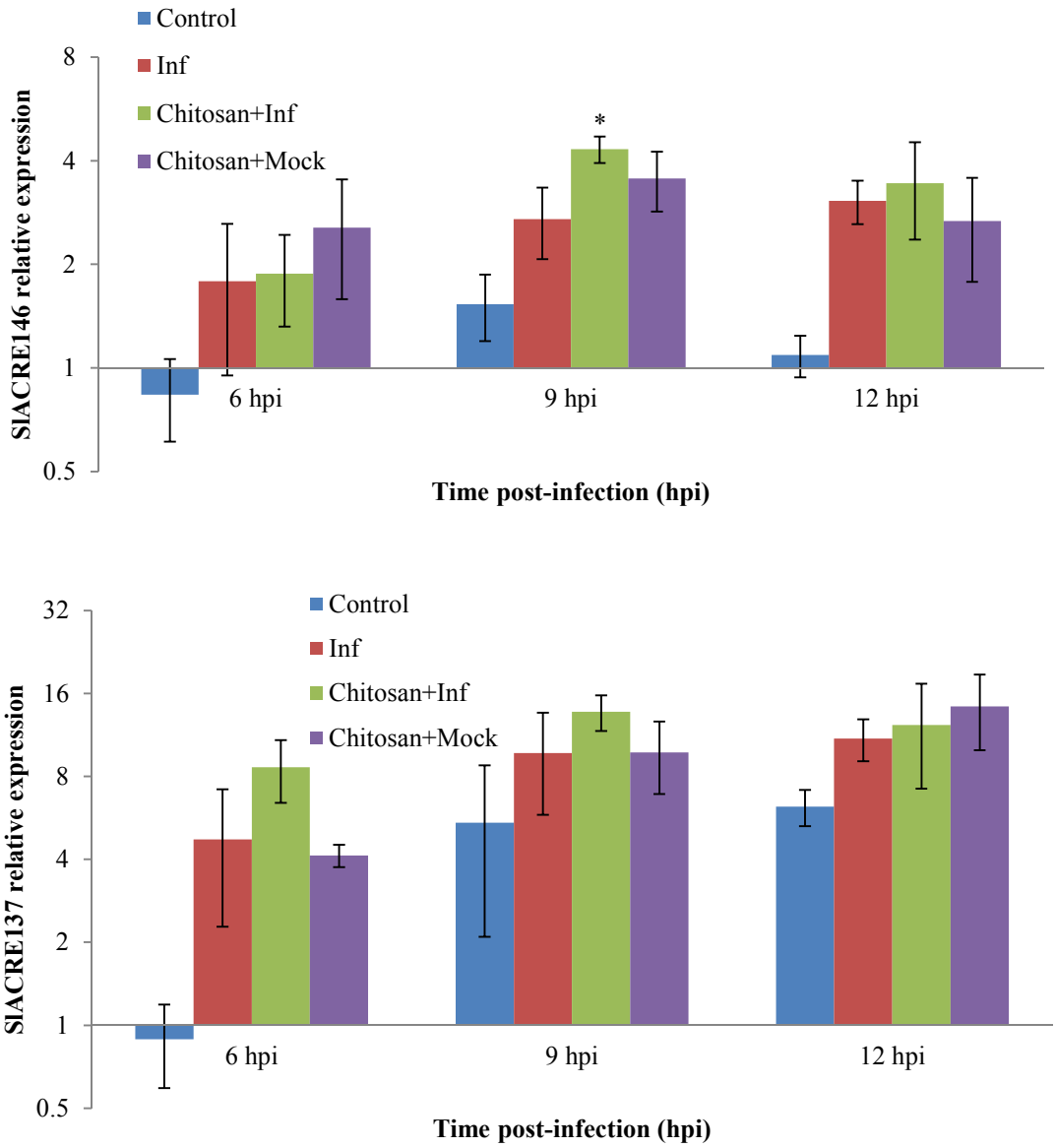


Figure 4.2 Relative expression of *SlACRE75*, *SlACRE180*, *SlACRE146* and *SlACRE137*. Data shown are the means of 4 biological replicates from the normalized values obtained by ANOVA-Benjamini Hochberg (BH) ($p < 0.05$) from the Microarray. Relative expression folds (Log2) were calculated with control (ddH_2O-treated and mock inoculated) samples. Asterisks indicate differential expression (2-way ANOVA P-value ≤ 0.05, Benjamini-Hochberg-corrected).

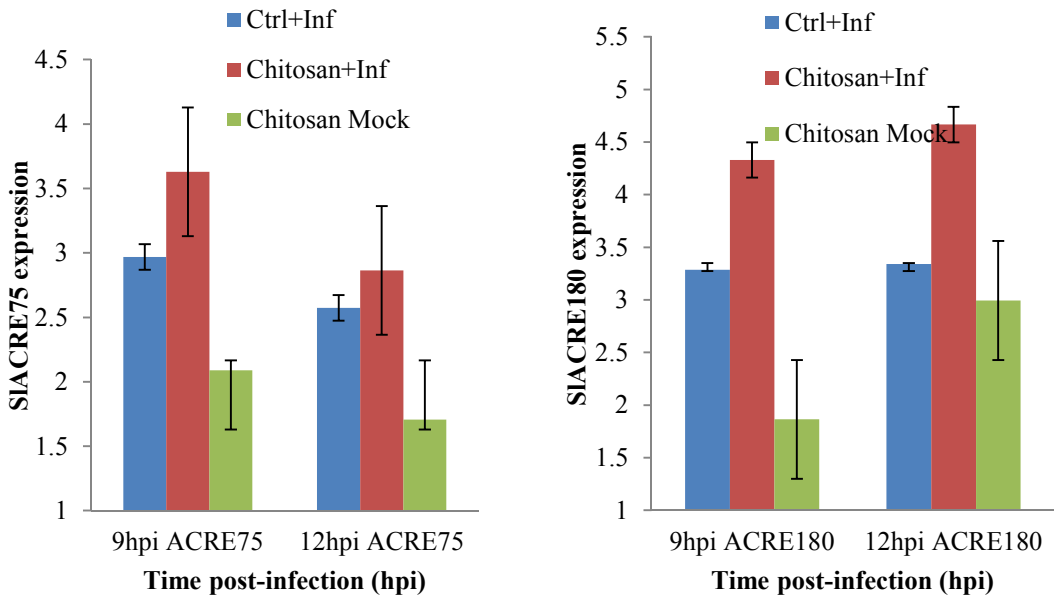
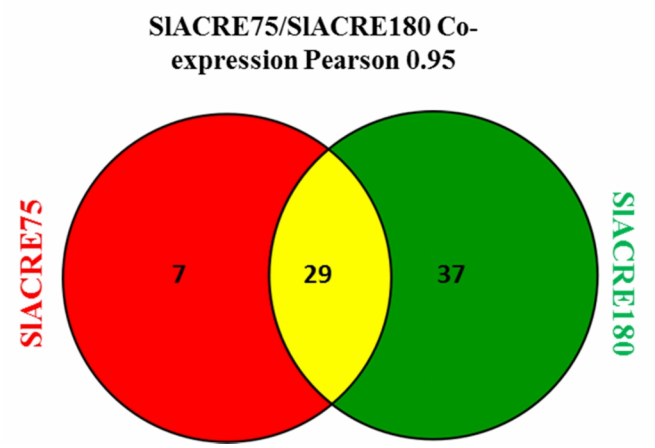


Figure 4.3 *SlACRE75* and *SlCRE180* relative expression values obtained by normalized gene expression relative to control (ddH_2O-treated and mock inoculated) by qRT-PCR (fold change, Log2). The data shown are the means of three biological replicates ± standard error of the mean (SEM). Ctrl+Inf, untreated and *B. cinerea*-infected plants; chitosan-Mock, chitosan-treated and non-infected plants; chitosan+Inf, chitosan-treated and infected plants were compared to control (ddH_2O-treated and mock inoculated) expression.

4.3.2 Co-expression analysis of *SlACRE75* and *SlACRE180*

SlACRE180 and *SlACRE75* fulfilled the priming phenotype requirements, being differentially expressed only by Chito+Inf, triggered by chitosan mock (non-infected) and they were primed (expressed earlier and stronger by Chito+Inf in comparison to Inf) (Figure 4.2). Furthermore, Pearson correlation analysis ($P \leq 0.05$) for *SlACRE75* and *SlACRE180* plotted against the DEGs list within the ANOVA (Figure 3.2), revealed that both genes are co-expressed, *SlACRE75* had 36 genes co-regulated and *SlACRE180* had 66, all of them were DEGs and they shared 29 DEGs including themselves (Figure 4.4). Some of 29 co-expressed genes with both *SlACRE75* and *SlACRE180* include various calmodulin genes, calcium-transporting ATPase, exocyst protein, harpin-induced protein, gibberellin 2-oxidase, auxins, *BCS1*, WRKYs, matrix metalloproteinase (*Sl2-MMP*) and a receptor-like kinase (Figure 4.4b).

(a)



(b)

Functional description	Genebank Accession
Calmodulin	Solyc07g053050.1.1
Avr9/Cf-9 rapidly elicited protein 180	Solyc08g016150.1.1
Calcium-transporting ATPase 1	Solyc02g064680.2.1
Os03g0816700 protein	Solyc10g085420.1.1
WRKY transcription factor 11	Solyc08g006320.2.1
Response regulator 5	Solyc02g071220.2.1
Os07g0175100 protein	Solyc12g009000.1.1
Exocyst complex protein EXO70	Solyc11g073010.1.1
Os03g0169000 protein	Solyc09g011860.2.1
cDNA clone J100026I16 full insert sequence	Solyc04g007580.1.1
Receptor-like kinase	Solyc02g089900.1.1
Mitochondrial carrier family	Solyc11g010500.1.1
Matrix metalloproteinase	Solyc04g005040.1.1
Avr9/Cf-9 rapidly elicited protein 75	Solyc11g010250.1.1
Calmodulin-like protein	Solyc02g094000.1.1
Harpin-induced protein-like	Solyc10g081980.1.1
Serine/threonine protein kinase family protein	Solyc04g007390.2.1
Gibberellin 2-oxidase	Solyc01g079200.2.1
Auxin-regulated protein	Solyc06g075690.2.1
CHP-rich zinc finger protein-like	Solyc02g068680.1.1
Calmodulin-binding protein	Solyc03g119250.2.1
Receptor-like protein kinase	Solyc02g080040.2.1
Unknown Protein	Solyc05g055080.1.1
WRKY transcription factor-30	Solyc08g082110.2.1
BCS1 protein-like protein	Solyc03g033770.1.1
WRKY transcription factor	Solyc08g008280.2.1

Calmodulin-like protein	Solyc02g088090.1.1
Tobacco rattle virus-induced protein variant 2	Solyc07g056600.1.1

Figure 4.4 **(a)** Venn diagram illustrating the number of common and specific genes co-regulated with *SlACRE75* (red circle) and *SlACRE180* (green circle) (Pearson correlation test $P \leq 0.05$). **(b)** Genes in table represent all 29 genes co-expressed with *SlACRE75* and *SlACRE180*. The 29 genes were included in the ANOVA-Benjamini Hochberg corrected.

4.3.3 Chitosan-induced gene expression in *Nicotiana benthamiana*: ACRE genes role on chitosan-primed plants against *Botrytis cinerea*

N. benthamiana is a plant from the Solanaceae family considered to be a model organism for functional studies, which has been extensively used to investigate host and pathogen gene functionality (Rowland et al. 2005; Li, Zhang, et al. 2014; X. Li et al. 2015; D. Li et al. 2015; McLellan et al. 2013; Ishihama et al. 2011; Peenková et al. 2011). *N. benthamiana* is also a susceptible host for *B. cinerea*, which makes it a suitable candidate to investigate plant-*B. cinerea* interaction further. The James Hutton Institute has a facility specialized for production of high-quality *N. benthamiana* wild-type and reporter lines (organelle marker fluorescence protein-tagged) for functional analysis.

4.3.3.1 *NbACRE75* and *NbACRE180* expression dynamics in *Nicotiana benthamiana* against *Botrytis cinerea*

In order to test the ability of chitosan to prime *N. benthamiana* defences against *B. cinerea*, the expression profile of *SlACRE75* and *SlACRE180* orthologues (subsequently termed *NbACRE75* and *NbACRE180*) and their putative role in chitosan-treated *N. benthamiana* plants infected with *B. cinerea* was investigated.

Both genes were induced by *B. cinerea* infection (Inf), as chitosan-treated + non infected (mock) plants did not trigger their expression. *NbACRE75* was triggered at 24 hpi for both Inf and primed by chitosan (Chitosan+Inf), whilst *NbACRE180* was induced from 6 hpi and it displayed a chitosan-primed phenotype at 24 hpi (Chitosan+Inf) (Figure 4.5). Thus, chitosan was able to prime both genes for a stronger expression at 24 hpi.

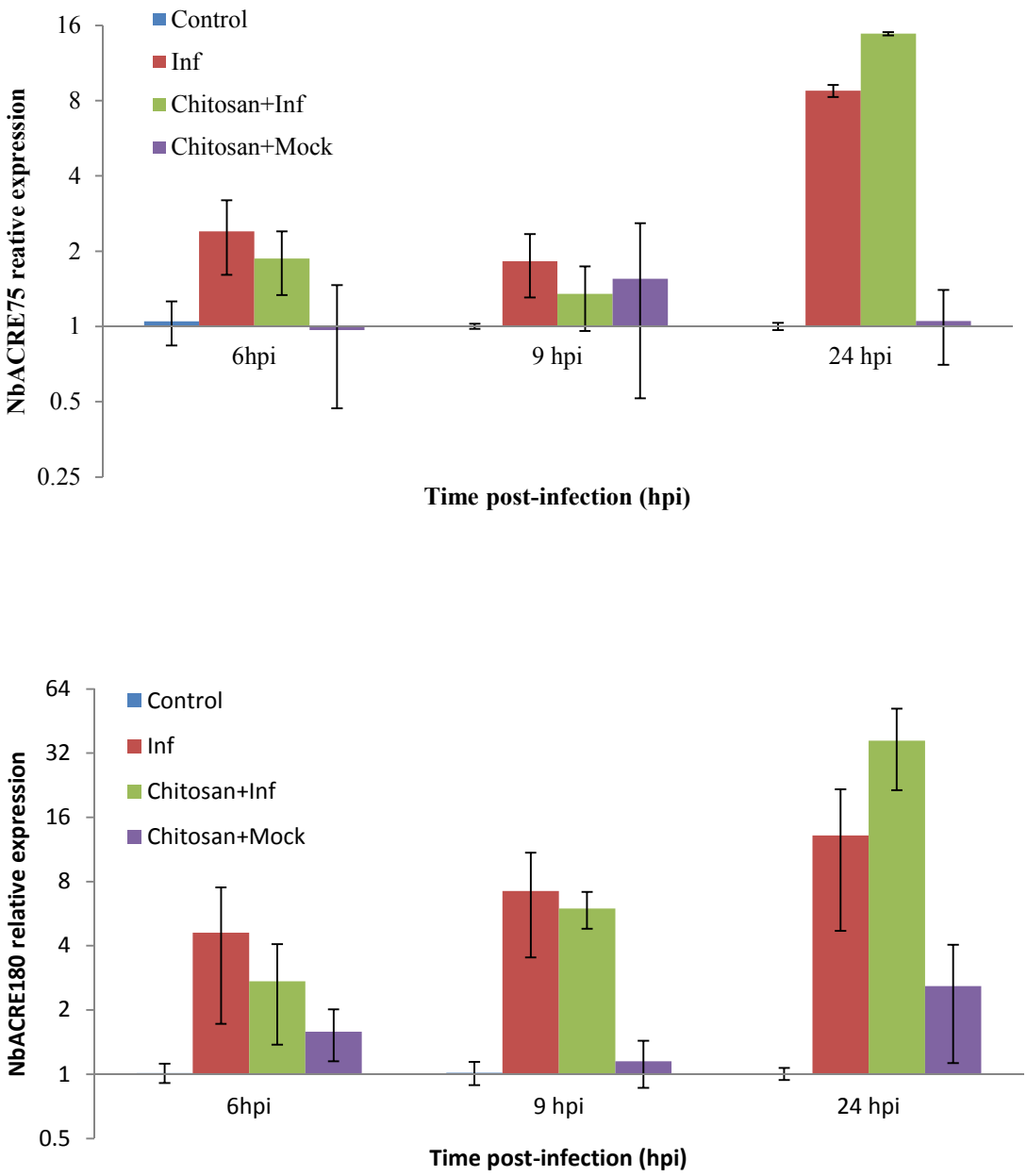
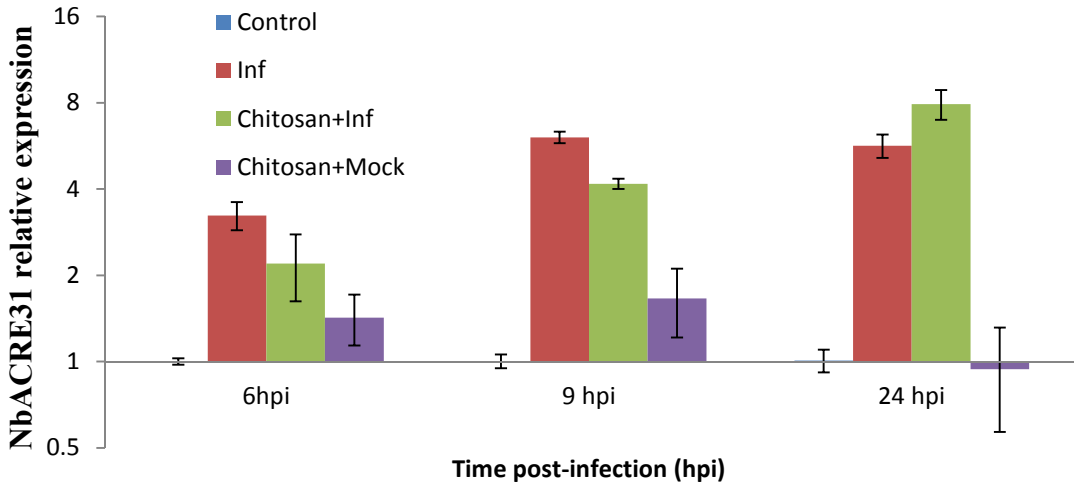


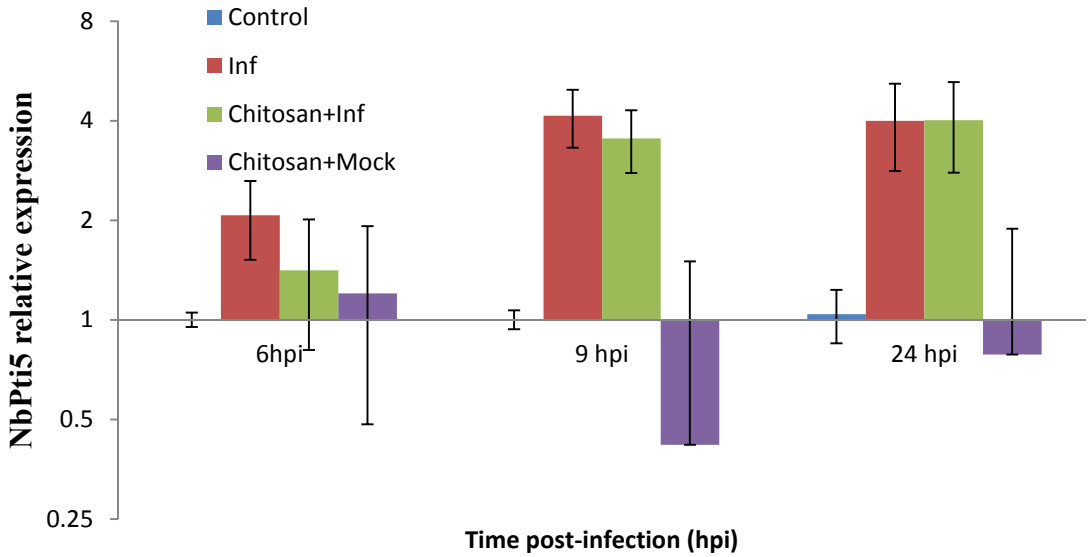
Figure 4.5 *NbACRE75* and *NbACRE180* relative expression. Fold change (Log2) values obtained by qRT-PCR. The data shown are the means of three biological replicates ± standard error of the mean (SEM). Relative expression folds (Log2) were calculated relative to control (ddH_2O-treated and mock inoculated) samples. Inf, ddH_2O-treated and *B. cinerea*-infected plants; Chitosan+Mock, chitosan-treated and non-infected/mock inoculated plants; Chitosan+Inf, chitosan-treated and infected plants.

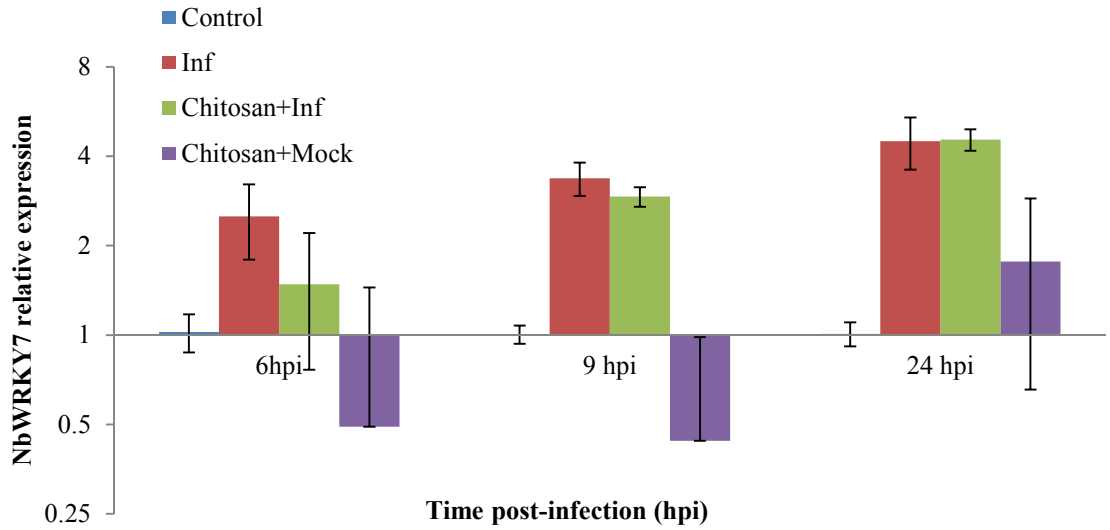
4.3.3.2 PTI marker genes role in chitosan-priming *Nicotiana benthamiana* defences against *Botrytis cinerea*

The expression of marker genes involved in the PTI response to pathogen attack, such as *NbACRE31*, *NbPti5*, *NbWRKY7* and *NbWRKY8* (Sonnewald et al. 2012; Boevink et al. 2016; Ishihama et al. 2011; Adachi et al. 2016) was studied to investigate further chitosan-priming properties in a tobacco relative, *N. benthamiana*, against *B. cinerea*.

All genes except *NbWRKY8* had a similar expression pattern among all treatments (Figure 4.6), being induced at 6 hpi and progressively increased their expression during the infection with a similar expression between ddH_2O-treated + *B. cinerea*-infected samples (Inf) and chitosan-treated + infected (Chitosan+Inf). Moreover, *NbACRE31* expression followed a similar pattern than *NbACRE75* and *NbACRE180* (Figure 4.5) in that it was highly induced at 24 hpi and primed by chitosan (Chitosan+Inf). In contrast, *NbWRKY8* expression differed from the other genes as it was highly induced and primed by chitosan at all time points (Figure 4.6). *NbWRKY8* expression was higher in chitosan-treated without infection (Chitosan+Mock) at 6 hpi whereas Inf and Chitosan+Inf plants up-regulation was reduced, presumably by the pathogen. At 9 hpi, *NbWRKY8* was primed by chitosan as it was highly up-regulated by Chitosan+Inf in comparison with Inf and Chitosan+Mock, whilst at 12 hpi it was up-regulated by both chitosan-related treatments (Chitosan+Inf and Chitosan+Mock) and with a lower expression in Inf plants, which again suggests a *B. cinerea*-caused reduction of *NbWRKY8* expression (Figure 4.6).




Control
Inf
Chitosan+Inf
Chitosan+Mock
NbPti5 relative expression
8
4
2
1
0.5
0.25
6hpi
9 hpi
24 hpi
Time post-infection (hpi)


Control
Inf
Chitosan+Inf
Chitosan+Mock
NbWRKY7 relative expression
8
4
2
1
0.5
0.25
6hpi
9 hpi
24 hpi
Time post-infection (hpi)

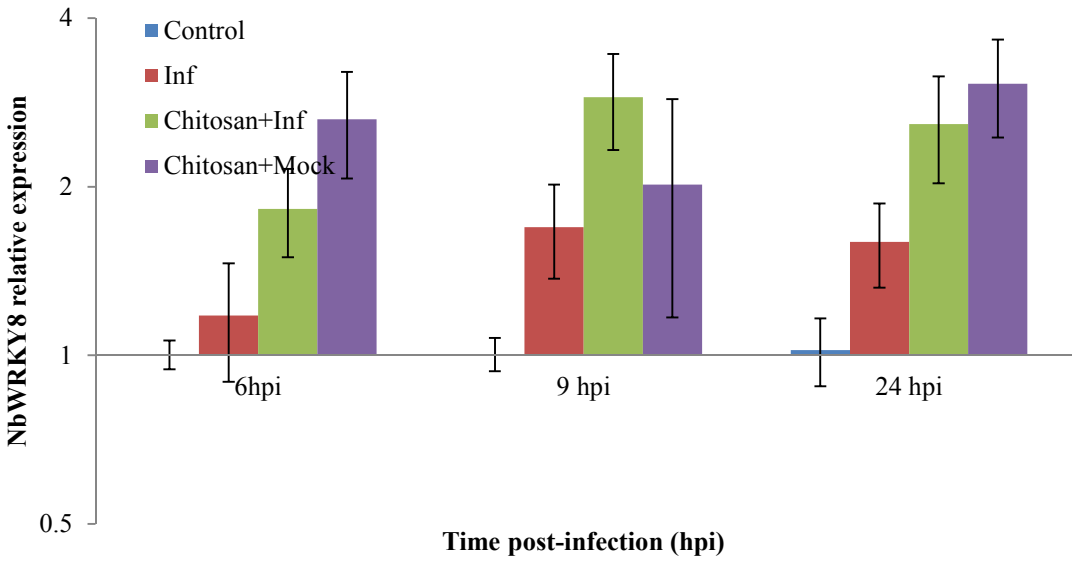


Figure 4.6 *NbACRE31*, *NbPti5*, *NbWRKY7* and *NbWRKY8* relative expression. Fold change (Log2) values obtained by qRT-PCR. The data shown are the means of three biological replicates ± standard error of the mean (SEM). Inf, ddH_2O-treated and *B. cinerea*-infected plants; Chitosan+Mock, chitosan-treated and non-infected plants; Chitosan+Inf, chitosan-treated and *B. cinerea*-infected plants.

4.3.4 Transient overexpression of *ACRE75* and *ACRE180* on *Nicotiana benthamiana* against *Botrytis cinerea*

This Chapter has shown that *SlACRE75*, *SlACRE180*, *NbACRE31*, *NbACRE75* and *NbACRE180* genes are triggered by chitosan (Chito+Mock) and *B. cinerea* (Inf) and their expression is increased/primed by chitosan after the infection (Chito+Inf) in tomato and *N. benthamiana* (Figures 4.3, 4.4). Moreover, both genes seemed to be co-regulated and involved in defence-related responses (Figure 4.4). Thus, it was hypothesized that ACRE75 and ACRE180 might be important for the successful or failure outcome of the host-*B. cinerea* interaction. Functional analysis was performed to investigate tomato and *N. benthamiana ACRE75* and *ACRE180* roles in resistance against fungal necrotroph *B. cinerea*.

Hence, using transient overexpression approach (see M&Ms), the 4 proteins, SlACRE75, SlACRE180, NbACRE75 and NbACRE180, and a GFP-non-protein/ empty vector (control) were agroinfiltrated into leaves of *N. benthamiana* plants and subsequently challenged with *B. cinerea*.

The transient overexpression was repeated twice with similar results, where all proteins contributed to significantly reduce disease compared to GFP-empty vector (Figure 4.7

(b) and (c) represent 2 independent experiments). Moreover, in the second assay, in order to investigate whether SlACRE75-SlACRE180 co-expression (Figure 4.4) may have an add-up effect and thus provide a stronger protection than the single ACRE75 or ACRE180 overexpression on the resistance against *B. cinerea*, both proteins SlACRE75-SlACRE180 were coinfiltrated (Combo 1-2) as well as NbACRE75-NbACRE180 (Combo 3-4) (Figure 4.7).

SlACRE75-, SlACRE180-, NbACRE75- and NbACRE180-infiltrated *N. benthamiana* plants significantly decreased *B. cinerea* necrotic lesion progress in comparison with GFP-infiltrated (empty vector/ negative control) (Figure 4.7 (a), (b) and (c)). In contrast, the combination of SlACRE75-SlACRE180 (Combo 1-2) treatment did not significantly decrease lesion expansion (Figure 4.7 (c)). Moreover, protein stability *in planta* and size was confirmed by protein immunoblot analysis of the fusion protein (GFP-ACRE) (Figure 4.7d).

These data demonstrate that transient overexpression of SlACRE75, SlACRE180, NbACRE75 and NbACRE180 in *N. benthamiana* plants conferred an increased resistance against *B. cinerea* which resulted into a decreased disease phenotype.

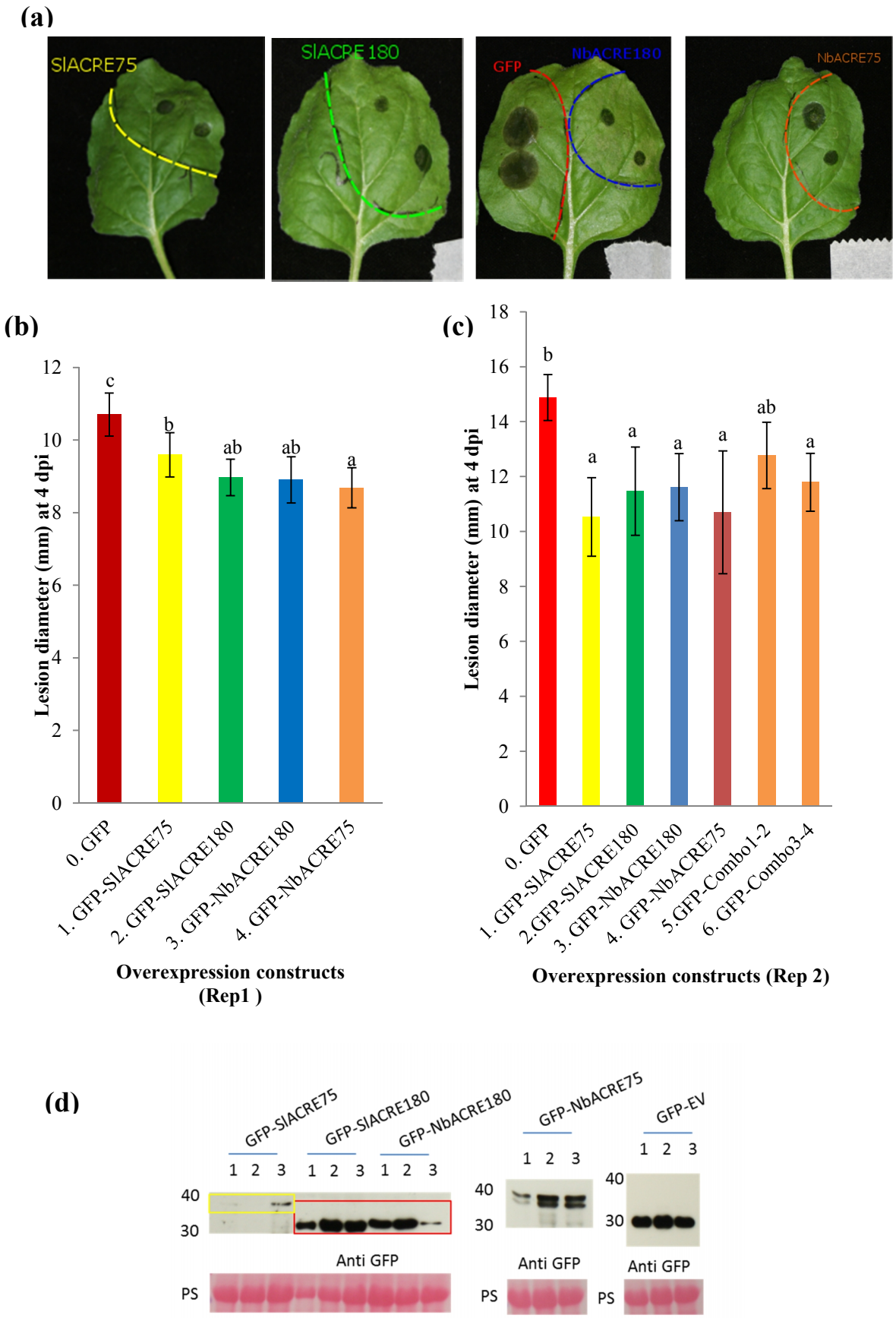


Figure 4.7 Transient expression of constitutively active SlACRE75, SlACRE180, NbACRE75 and NbACRE180 in *N. benthamiana* resulted in increased disease resistance against *B. cinerea*. **(a)** Disease symptom. **(b)** and **(c)** Lesion size (mm) and **(d)** expression of proteins by immunoblot analysis of GFP-

SlACRE75, GFP-SlACRE180, GFP-NbACRE75 and GFP-NbACRE180 fusion proteins in *N. benthamiana* leaves at 48 h after agroinfiltration. Three lanes represent 3 replicates per construct **(a)** GFP-SlACRE75, GFP-SlACRE180, GFP-NbACRE75, GFP-NbACRE180, and a GFP-non-protein/ empty vector (control) were transformed into *Agrobacterium tumefaciens* GV3101 strain and were agroinfiltrated into leaves of *N. benthamiana* plants. Opposite part of whole agroinfiltrated leaves were excised 2 days after agroinfiltration for pathogenicity test and subsequently challenged with *B. cinerea* suspension (2×10^4 spores/ mL). Photos were taken at 3 and 4 dpi. **(b,c)** Lesion size measurements were performed at 4 days post-infection (dpi) on 6 independent plants and 3 leaves/ construct to look for a resistance phenotype. Values presented are means ± SEM. Different letters indicate statistically significant differences (ANOVA $p < 0.01$ followed by Tukey's Post-hoc at 4 dpi). **(d)** Proteins were separated by SDS–PAGE and analysed by immunoblotting. A GFP-specific antibody was used for detection of GFP-fusion protein. Equal loading of total proteins was examined by Ponceau staining.

4.3.5 Subcellular localization of *SlACRE75*, *SlACRE180*, *NbACRE75* & *NbACRE180*

During this Chapter it has been shown *ACRE75* and *ACRE180* have key implications in chitosan-priming for resistance tomato and *N. benthamiana* against *B. cinerea*. Thus, because of the importance shown of *SlACRE75, NbACRE75, SlACRE180* and *NbACRE180* in plant defence response against the fungal necrotroph and since ACRE genes are poorly studied, *ACRE75* and *ACRE180* in particular, this study required investigation to find and confirm cell organelle localization.

The subcellular localization of *SlACRE75*, *SlACRE180*, *NbACRE75* and *NbACRE180* was investigated through a transient overexpression approach. The constructs were co-infiltrated with different markers, such as pFlub vector, which contains a cassette with an RFP-peroxisome tagged construct (McLellan et al. 2013) and they were infiltrated into *N. benthamiana* reporter lines CB157 (nucleus mRFP marker) and CB172 (ER mRFP marker) to confirm organelle localization. Furthermore, no signal peptides were found by prediction for any of the four proteins (SignalP 4.1), which suggests an intracellular function.

As shown in Figures 4.8 and 4.11, the GFP-SlACRE75 and GFP-NbACRE75 fusions accumulated exclusively in the nucleus and nucleolus of *N. benthamiana* cells, whereas the GFP protein alone accumulated in both the cytoplasm and the nucleus (Figure 4.12), demonstrating that both *SlACRE75* and *NbACRE75* proteins are localized in the nucleus of cells.

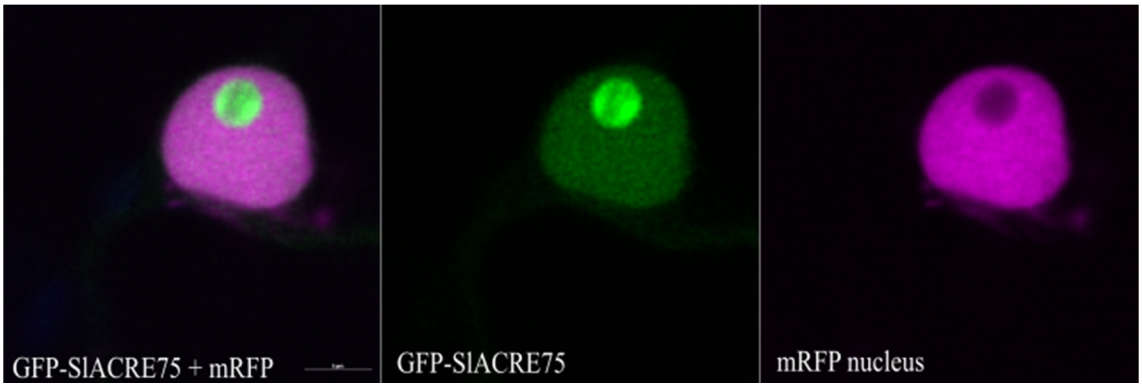


Figure 4.8 Subcellular localization of *SlACRE75* when transiently expressed in *N. benthamiana* leaves. The GFP:SlACRE75 construct was transiently expressed through agroinfiltration in *N. benthamiana* CB157 reporter line (with nucleus mRFP marker) leaves and green fluorescence of the GFP was viewed with a confocal laser microscope. The cells were examined under merged image (left), GFP/green fluorescence (middle), and as mRFP/red fluorescence (right) showing the nuclear localization of the GFP-SlACRE75 fusion protein. Scale bar = 10 μM.

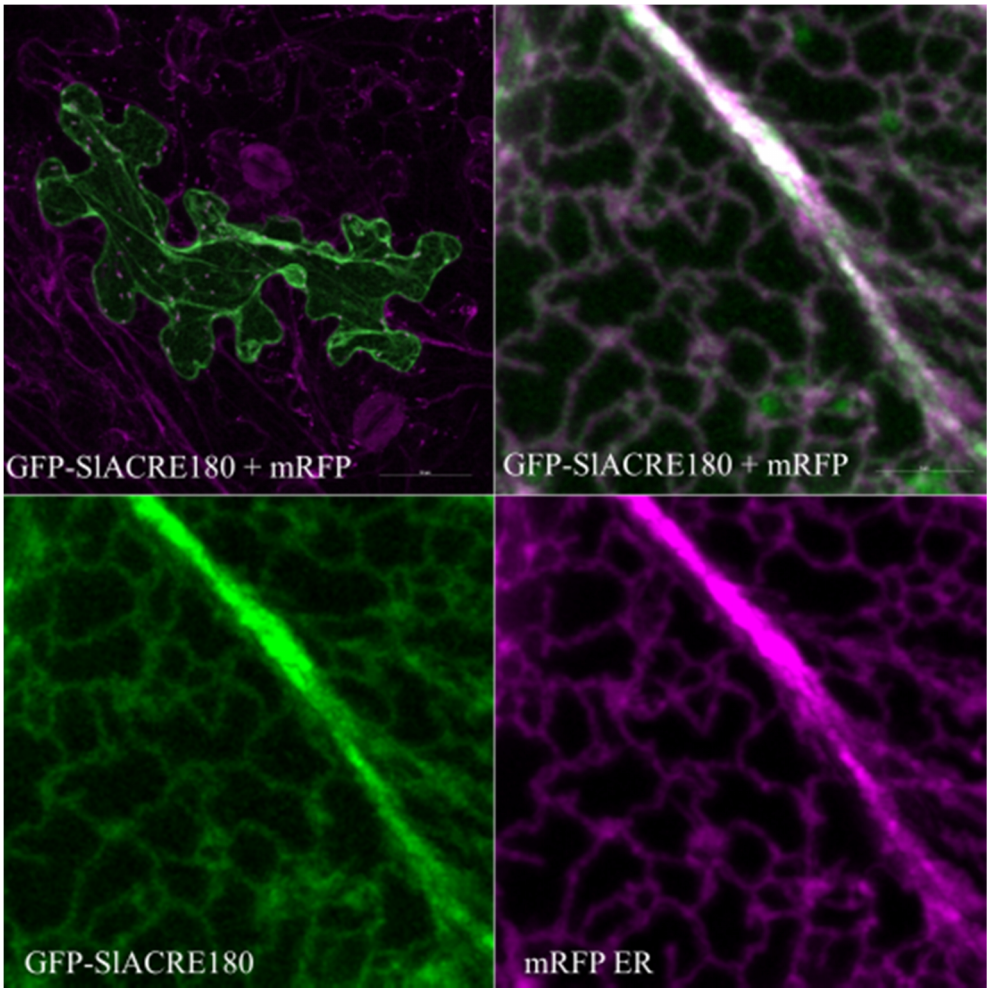


Figure 4.9 Subcellular localization of SlACRE180 when transiently expressed in *N. benthamiana* leaves. The GFP:SlACRE180 construct was transiently expressed through agroinfiltration in *N. benthamiana*

CB172 reporter line (with ER mRFP marker) leaves and green fluorescence of the GFP was viewed with a confocal laser microscope. The cells were examined under the merged fluorescence (upper left), closer merged (upper right), GFP/green (lower left), and mRFP/red fluorescence (lower right), showing the ER localization of the GFP-SlACRE180 fusion protein. Scale bar = 10 μM (upper right), 50 μM (upper left).

Furthermore, *SlACRE180* and *N. benthamiana ACRE180* orthologue were also agroinfiltrated and subsequently evaluated for confocal microscopy. As Figure 4.9 shows GFP-SlACRE180 fusion accumulated exclusively in the ER, however GFP-NbACRE180 fusion accumulation was exclusively accumulated in the peroxisomes (Figure 4.10) whereas GFP protein alone again did not follow a discreet localization (Figure 4.12), which demonstrates that *SlACRE180* is localized in the ER and *NbACRE180* in the peroxisomes.

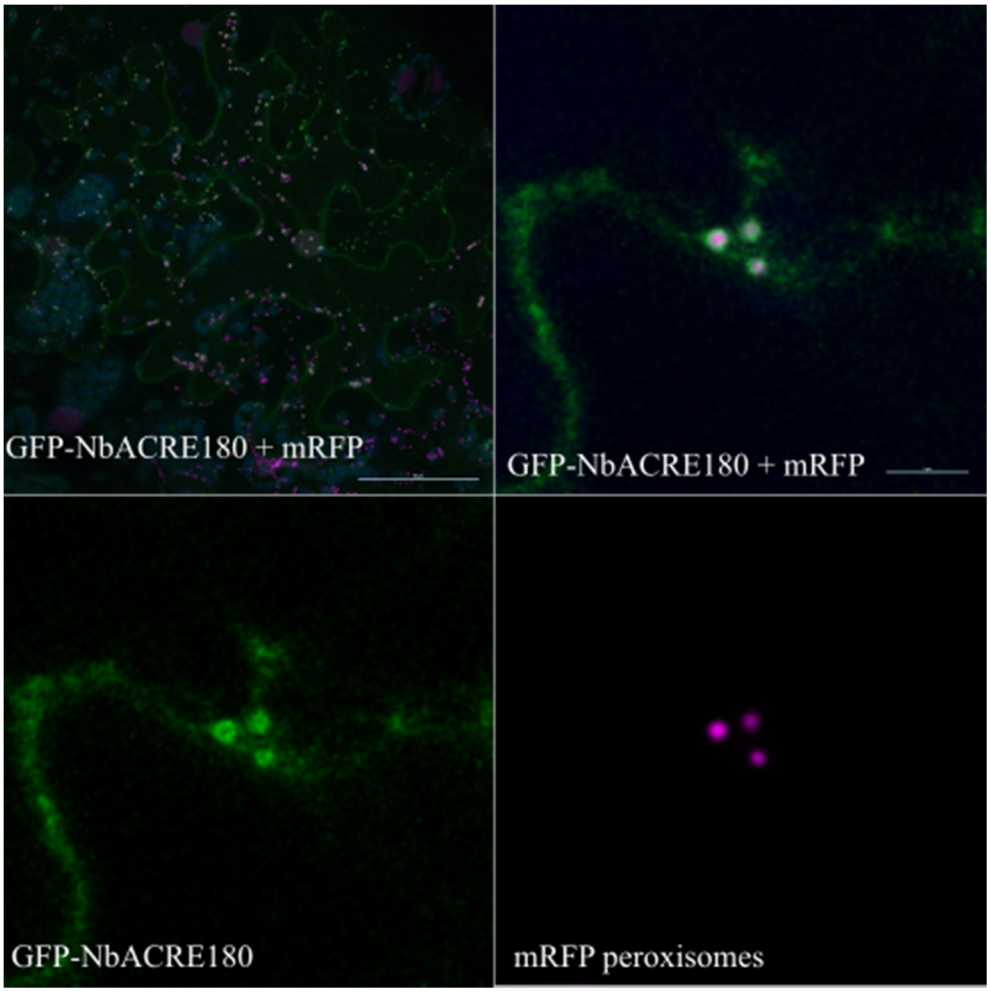


Figure 4.10 Subcellular localization of *SlACRE180* when transiently expressed in *N. benthamiana* leaves. The GFP:NbACRE180 construct was co-infiltrated with pFlub vector (RFP-peroxisome tagged marker), and it was transiently expressed through agroinfiltration in *N. benthamiana* leaves and green fluorescence

of the GFP was viewed with a confocal laser microscope. The cells were examined under the merged fluorescence (upper left), closer merged (upper right), GFP/green (lower left), and mRFP/red fluorescence (lower right) showing the peroxisome localization of the GFP-NbACRE180 fusion protein. Scale bar = 10 µM (upper right), 50 µM (upper left).

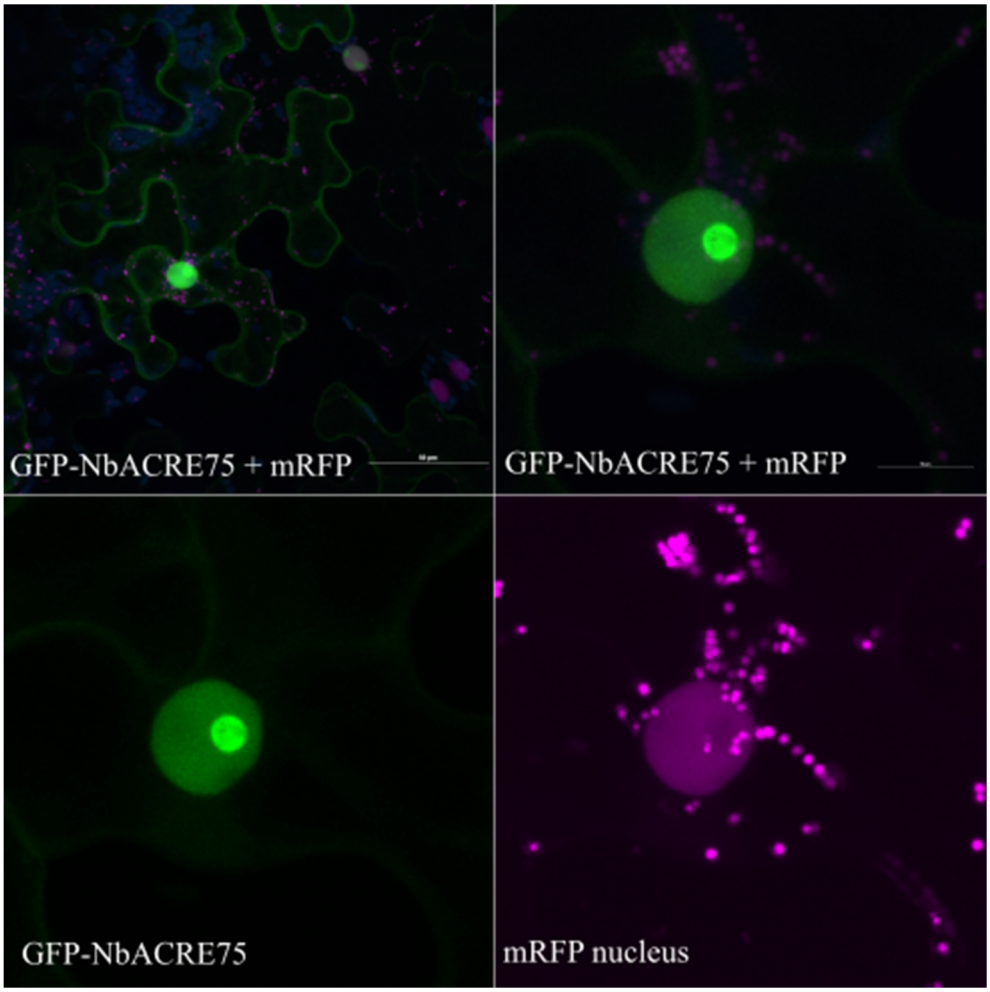


Figure 4.11 Subcellular localization of *NbACRE75* when transiently expressed in *N. benthamiana* leaves. The GFP:NbACRE75 construct, co-infiltrated with pFlub vector (RFP-peroxisome tagged marker), was transiently expressed through agroinfiltration in *N. benthamiana* leaves and green fluorescence of the GFP was viewed with a confocal laser microscope. The cells were examined under the merged fluorescence (upper left), GFP/green (lower left) and mRFP/red (lower right) showing the nuclear localization of the GFP-NbACRE75 fusion protein. Scale bar = 10 µM (upper right), 50 µM (upper left).

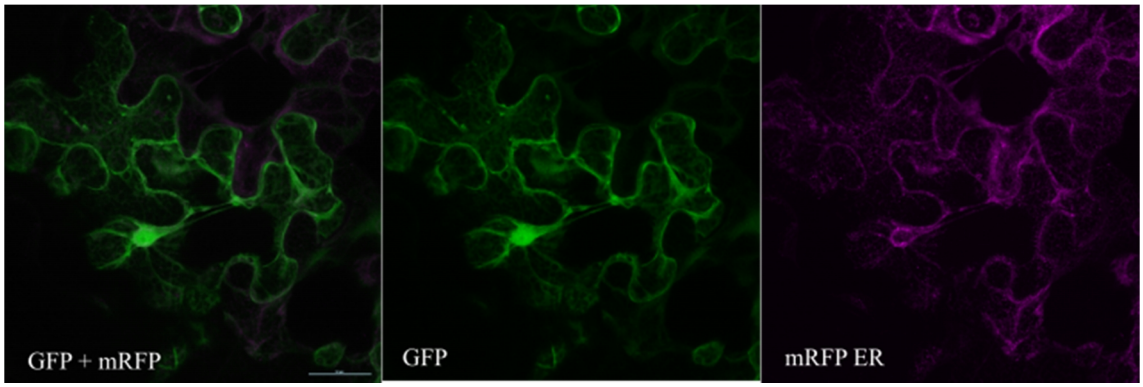


Figure 4.12 Subcellular localization of pB7WGF2:35S:GFP (empty vector) when transiently expressed in *N. benthamiana* leaves. Free GFP construct was transiently expressed through agroinfiltration in *N. benthamiana* leaves and green fluorescence of the GFP was viewed with a confocal laser microscope. The cells were examined under the merged fluorescence (left), GFP/green fluorescence (middle) and mRFP/red fluorescence (right) showing the diffused GFP-empty vector (control plasmid). Scale bar = 50 µM.

4.3.6 Characterisation of *ACRE75* and *ACRE180 Arabidopsis thaliana* overexpression stable lines resistance against *Botrytis cinerea*

It has been shown that transient overexpression of *SlACRE75*, *SlACRE180*, *NbACRE75* and *NbACRE180* in the model plant *N. benthamiana* conveys enhanced resistance to *B. cinerea*. Moreover, *A. thaliana* plants lack on homologous ACRE75 and ACRE180 proteins from tomato (BLAST query on TAIR10 Proteins and multialignment, data not shown). To further analyse *ACRE75* and *ACRE180* biological functions and and to confirm their role in plant resistance against *B. cinerea*, *A. thaliana* plants were transformed to constitutively overexpress SlACRE75, SlACRE180, NbACRE180 and NbACRE75 proteins.

All the transgenic plants showed no visible alteration in growth, development or morphology from wild-type plants (Col-0). Five-week-old plants were infected with *B. cinerea* by drop inoculating leaves and disease was scored at 3 and 6 dpi (Figure 4.13). Transgenic SlACRE75-, SlACRE180-, NbACRE180- and NbACRE75-overexpression plants showed an enhanced resistance phenotype and significantly decreased *B. cinerea* lesions in comparison to Col-0 and GFP-EV controls (Figure 4.13). Furthermore, SlACRE75- and its homolog NbACRE75-overexpression plants showed a stronger resistance to *B. cinerea* than SlACRE180- and NbACRE180-overexpression lines at 3 and 6 dpi (Figure 4.13).

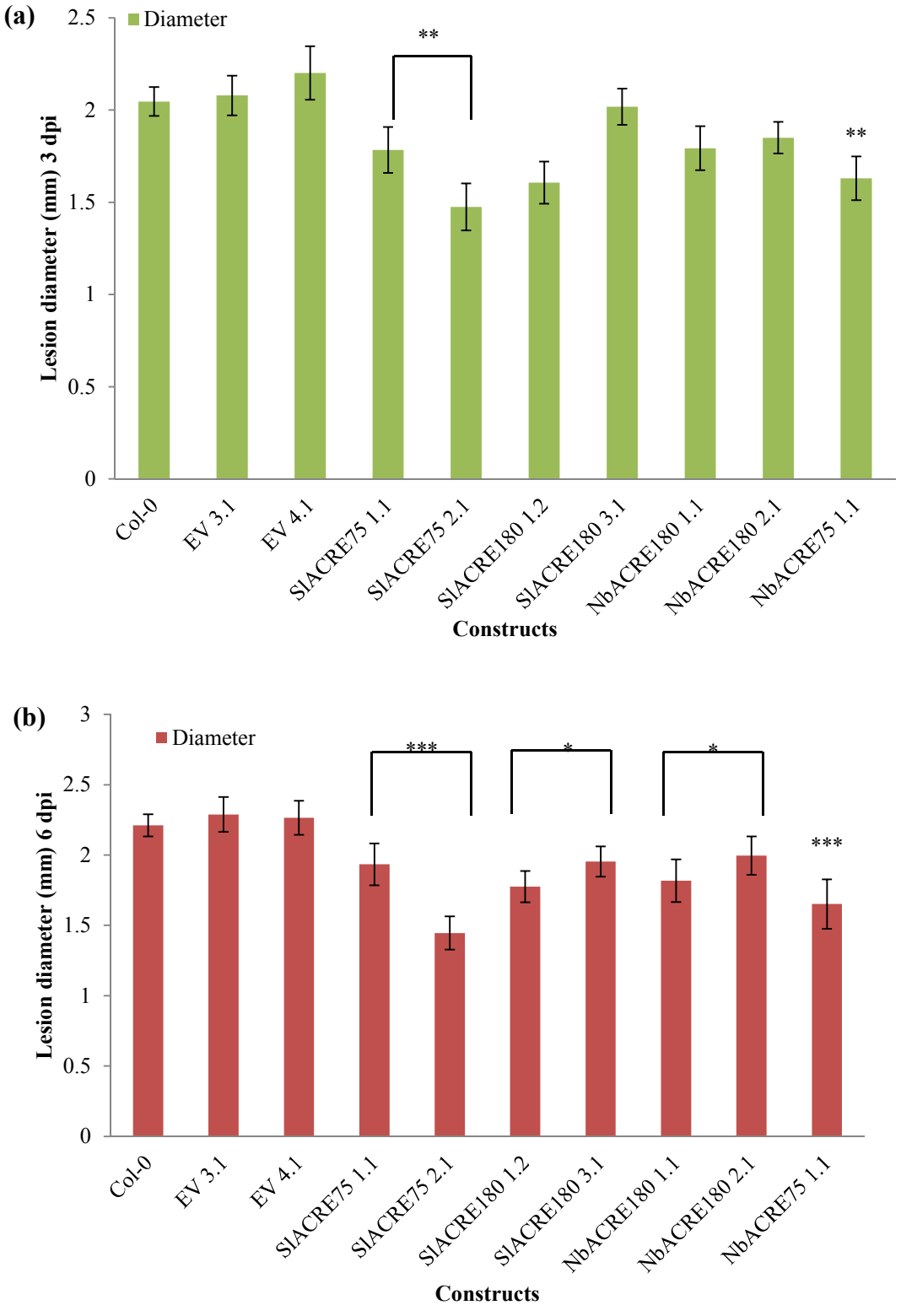


Figure 4.13. *A. thaliana* transformed overexpression stable SlACRE75, SlACRE180, NbACRE75 and NbACRE180 lines resulted in increased disease resistance against *B. cinerea*. Plants were infected with *B. cinerea* spores by drop inoculating leaves with an inoculum containing $5*10^5$ spores/ mL. Lesion sizes were measured at 3 and 6 days after inoculation (dpi) on 8-16 independent plants/ construct. Values presented are means ± SEM. Asterisks indicate statistically significant differences (* $p < 0.05$, ** $p <$

0.01; *** $p < 0.001$). **(a)** Significant differences among constructs at 3 dpi ($p = 0.001$). Contrasts with Col-0 show EV $p = 0.494$; SlACRE75 $p = 0.002$; SlACRE180 $p = 0.069$; NbACRE180 $p = 0.086$; and NbACRE75 $p = 0.002$. **(b)** Significant differences among constructs at 6dpi ($p < 0.001$). Contrasts with Col-0 show EV $p = 0.661$; SlACRE75 $p < 0.001$; SlACRE180 $p = 0.015$; NbACRE180 $p = 0.035$; and NbACRE75 $p < 0.001$.

4.4 DISCUSSION

Commercial crops are susceptible to necrotrophic pathogens, such as *B. cinerea*. Many studies have shown the complex infection strategy of *B. cinerea* (Espino et al. 2010; Van Kan 2000; Gonzalez-Rodriguez et al. 2014; González-Fernández et al. 2015), from which host plants are usually overwhelmed and fail to stop the infection progress. However, plants fight back with the activation of multifaceted signalling pathways that, if fine-tuned, could potentially contribute to the reduction or termination of fungal necrotroph infection and ultimately prevent disease. In chapter 3 it was revealed how a well-known PAMP/MAMP can function as a priming agent and hence it primes tomato plants for a more robust and efficient induced resistance during the initial stages of the infection. In the fight against pathogens, the initial moments of the infection can be decisive for the plant to become diseased or develop any type of resistance.

This study has identified two novel genes that are involved in basal and elicitor-induced resistance against fungal necrotroph *B. cinerea*. Avr9/Cf-9 rapidly elicited (ACRE) genes, which can be triggered 15 to 30 min after challenge, are crucial for the initial defence responses against biotic stress (Durrant et al. 2000). Four ACRE novel genes were identified that play a putatively role in tomato and *N. benthamiana* resistance against *B. cinerea*. Interestingly, in tomato three of these genes were primed by chitosan (Chitosan+Inf) (Figure 4.2) and 2 of them (*SlACRE75* and *SlACRE180*) were co-regulated and shared 29 differentially expressed genes (DEGs) also co-expressed (Figure 4.4). Some of these 29 DEGs have been reported to be involved in plant defence against various pathogens including *B.cinerea*, such as Solyc04g005040.1.1, a matrix metalloproteinase (*Sl2-MMP*), which was shown to be induced by *B. cinerea* and *Pseudomonas syringae* pv. tomato (Pst) DC3000 together with *Sl3-MMP*, which contributes to tomato resistance to both pathogens (Li et al. 2015); a harpin-induced protein; harpin can have a positive effect in protecting pepper and *A. thaliana* against *B. cinerea*, *Hyalonoperonospora arabidopsidis* and Pst (Tezcan et al. 2013; Dong et al.

1999); and exocyst complex subunit protein *EXO70*, which has been found to play a role in cell-wall papillae formation against pathogen attack (Peenková et al. 2011).

Interestingly, four calmodulin genes and calcium-transporting *ATPase 1* were coexpressed with *SlACRE75*/*180*, which suggests a possible role of *SlACRE75* and *SlACRE180* in activating calcium-related signalling defences. Calcium is an important element that acts as a second messenger in plant development and it can mediate plant defences against biotic stresses (Gravino et al. 2015; Batistič & Kudla 2012). Furthermore, studies have shown that calcium-related genes can play a key role in defence against *B. cinerea*, such as the calmodulin gene *SlCaM2*, which was found to be a positive regulator of tomato resistance to *B. cinerea* (Peng et al. 2014) and calcium-dependent protein kinases (*AtCPK6*, *AtCPK7* and *AtCPK11*) which are involved in *A. thaliana* basal and induced resistance against *B. cinerea* (Gravino et al. 2015).

Another important finding was that chitosan was able to prime (Chitosan+Inf) *N. benthamiana* orthologues *NbACRE75*, *NbACRE180* and PTI-marker gene *NbACRE31* (Sonnewald et al. 2012) at 24 hpi (Figures 4.5 and 4.6), which suggests a possible extrapolation of chitosan-priming effect on ACRE genes on other Solanaceous plants. In contrast, chitosan did not prime two other PTI-marker genes (*NbPti5* and *NbWRKY7*), but it induced (Chitosan+Mock) and primed (Chitosan+Inf) *NbWRKY8*. *NbWRKY8* is the closest WRKY to *AtWRKY33* with a 48.2 % of amino acid identity (Adachi et al. 2016), a gene involved in *A. thaliana* resistance to *B. cinerea*. Furthermore, the *wrky33* mutant was impaired in JA-defence genes when infected by *B. cinerea* (Adachi et al. 2015), suggesting a possible involvement of *NbWRKY8* in chitosan-priming for JA-dependent defences (Figure 4.6).

ACRE genes can be expressed upon infection, wounding, treatment with elicitors, Avr proteins and pathogen/microbe-associated molecular patterns (PAMPS, MAMPs) (González-Lamothe et al. 2006; Boevink et al. 2016; Rowland et al. 2005). Many ACRE genes functions have been deciphered in previous studies (Durrant et al. 2000; González-Lamothe et al. 2006; Rowland et al. 2005), and they usually encode components of signalling pathways, including transcription factors, protein kinases, and ubiquitination pathway-related proteins, such as F-box and U-box proteins (González-Lamothe et al. 2006). Some ACRE genes have been associated to PAMP-triggered immunity (PTI) responses, such as *ACRE31* (Boevink et al. 2016; Sonnewald et al.

2012) while others, such as *ACRE74*, *ACRE276* and *ACRE189* are related to hypersensitive response (HR) (González-Lamothe et al. 2006; Sadanandom et al. 2012) as part of the effector-triggered immunity (ETI) response. However, *ACRE75* and *ACRE180* molecular functions still remain unknown. To date, no study has addressed the role of ACRE genes in plant defence against the fungal necrotroph *B. cinerea* although Hennin et al. 2002 showed that low doses of the Avr9 elicitor, corresponding to fungal biotroph *Cladosporium fulvum*, can delay *B. cinerea* development in *Cf9*tomato and *Sclerotinia sclerotium* in *Cf9*oilseed rape plants, independently to HR. The Avr9/Cf9 interaction triggers active oxygen species (AOS) and the expression of ACRE genes, but most of ACRE gene activation is not dependent of AOS (Durrant et al. 2000), which indicates that ACRE genes are involved in alternative defence pathways (Figure 4.1). Thus, a reverse genetics approach was required to determine the function of *SlACRE75*, *SlACRE180*, *NbACRE180* and *NbACRE75*. A gene silencing could be used to understand whether *SlACRE75* and *SlACRE180* determine whether chitosan is able to induce resistance in *slacre75* and *slacre180* KO lines, but successful tomato plants transformation can take up to 12 months. Therefore, *SlACRE75* and *SlACRE180 A. thaliana* orthologues were chosen as a faster and easier cloning model plant with a large mutant collection, a host of *B. cinerea* and chitosan induces resistance (see Chapter 2). This would have given the opportunity to study whether chitosan is able to induce resistance in *acre75* and *acre180* KO lines. However, no significant similarity was found in *A. thaliana* for *SlACRE180* nor *SlACRE75* CDS (NCBI Blast: nucleotide seq) and low similarity score was found for both proteins (unknown protein with 40 % identity and 53.5 score best hit for *SlACRE75* and unknown protein with 39% identity and 28.1 score for *SlACRE180*; NCBI Blast: protein and TAIR). Therefore, transient overexpression in *N. benthamiana* was chosen as various studies have shown this to be effective for proteins in *N. benthamiana* against *B. cinerea* (Li et al. 2014; Li et al. 2015; Li et al. 2014; Li et al. 2015; Liu et al. 2014). Functional analysis of tomato and *N. benthamiana ACRE75* and *ACRE180* revealed that transient overexpression of the four proteins significantly decreases *B. cinerea* disease without HR induction compared to the empty vector. This suggests an *ACRE75*/*180*-triggered defence pathway that does not require AOS/HR induction against *B. cinerea* in *N. benthamiana*, which might be beneficial for the plant as *B. cinerea* is well-known to manipulate its host defences and produce host cell death during the infection (Chen et

al. 2010; Sivakumaran et al. 2016; Asselbergh et al. 2007b). Moreover, Pearson co-expression analysis resulted in *SlACRE75* and *SlACRE180* being co-expressed and shared 29 DEGs including genes involved in defence responses, which could indicate a similar function or that both proteins may be involved in the same signalling pathway. The bHLH subgroup IIId transcription factors *bHLH3*, *bHLH13*, *bHLH14* and *bHLH17* were found to have a redundant function in repressing JA-mediated responses in *A. thaliana* (Song et al. 2013).

Thus, in order to study further ACRE75 and ARE180 molecular functions and to investigate whether these two proteins are part of the same signalling pathway or instead they share a similar function and whether this influences a stronger or weaker resistance phenotype, the four proteins were co-infiltrated (Figure 4.7 (c)) in *N. benthamiana* leaves. However, SlACRE75-SlACRE180 coinfiltration did not significantly decrease *B. cinerea* disease expansion, which suggests that ACRE75 and ACRE180 might be involved in the same pathway, as the lack of an additive effect of a double knock-out/over-expression usually indicates the 2 proteins concerned are in the same pathway. However, *A. thaliana* transgenic SlACRE75- and NbACRE75-overexpression lines showed a stronger resistance phenotype than SlACRE180- and NbACRE180-overexpression plants (Figure 4.13), which suggests different functions and confirms ACRE75 and ACRE180 positive roles in disease resistance against *B. cinerea*.

The four proteins do not contain signal peptides (SPs) (Appendix), which indicate that their function is either cytoplasmic or nuclear. Subcellular localization analysis showed that GFP-SlACRE75 and GFP-NbACRE75 were localized in the nucleus and nucleolus (Figures 4.8 and 4.11), while GFP-SlACRE180 was localized mainly in the ER (Figure 4.9). Nuclear localization of both tomato and *N. benthamiana* ACRE75 indicates a signalling role of this protein, as it is known that for the plant immune processes, such as PTI or ETI, the translocation of proteins, including receptor kinases, MAPK cascades or transcriptional activators, from the transmembrane to the nucleus is a crucial step for the onset of plant immune dynamics (Motion et al. 2015; Adachi et al. 2015). Moreover, previous analysis on *H. arabidopsidis*-infected *A. thaliana* plants have reported that the non-protein amino acid elicitor *β*-aminobutyric acid (BABA) forced the translocation of *IBI1* gene, an aspartyl-tRNA synthetase, from the ER into the cytoplasm, as part of its

priming process of *A. thaliana* against *H. arabidopsidis* (Luna et al. 2014), which open new insights to understand how priming agents influence cell signalling against pathogen attack. Thus, further experiments on chitosan-*ACRE75*/*180* mediated cell organelle translocation could help to further decipher chitosan priming effects on the plant cell.

Transcriptome analysis (Chapter 3) showed ethylene (ET) (synthesis, metabolism and ET-related transcriptional factors) together with jasmonic acid were both crucial hormone pathways triggered/primed by both chitosan and *B. cinerea*. Interestingly, recent studies have shown that ethylene perception and signalling takes place in the ER (Ju & Chang 2012) and the inactivation of the jasmonic acid bioactive compound jasmonoyl-L-isoleucine (JA-Ile), found to be a key hormone involved in chitosan priming for tomato resistance against *B. cinerea* (Figure 3.25), occurs in the ER (Koo et al. 2014), which demonstrates the importance of the ER in both these plant hormones (JA and ET) involved in resistance against *B. cinerea*. Finally, GFP-*NbACRE180* is localized in the peroxisomes; an essential organelle involved in oxidation processes, and therefore in the production of reactive oxygen species (ROS). Peroxisomes were found to be a target of *B. cinerea,* which manipulates its antioxidant balance to promote cell death (Kuzniak & Skłodowska 2005). Moreover, the β-oxidation of 12-oxo-phytodienoic acid (OPDA) into JA takes part in the peroxisome (Wasternack & Hause 2013; Guo et al. 2015), which again confirms the importance of JA pathway in chitosan-priming ACRE genes against *B. cinerea*.

4.5 CONCLUSION

Here, I report the use of a well-known PAMP/MAMP, chitosan, with a novel approach, as a priming agent. Chitosan was able to prime tomato and *N. benthamiana* plants for a more efficient and faster defence response through priming Avr9/Cf-9 rapidly elicited (ACRE) 75 and *ACRE180*, two genes that in tomato are co-regulated together with 29 genes, including calmodulin, receptor-like kinases, WRKYs and cell-wall-related genes such as *Sl2-MMP* and *EXO70*. Transient and constitutively overexpression of *SlACRE75*, *SlACRE180* and their *N. benthamiana* orthologues *NbACRE75* and *NbACRE180* resulted in increased resistance to *B. cinerea* and associated disease reduction. Furthermore, subcellular localization of the four proteins indicates that they are involved in intracellular signalling. These results demonstrated that both *ACRE75*

and *ACRE180* function as positive regulators of defence response against *B. cinerea* in tomato and *N. benthamiana*. Thus, *SlACRE75*/*180* might be part of or trigger a complex signalling pathway that includes intracellular transcriptional reprogramming. However, the biological functions of *ACRE75* and *ACRE180* need to be further investigated. Defence gene expression dynamics on ACRE KO mutants will be helpful in elucidation of the molecular mechanism of *SlACRE75* and *SlACRE180* in disease resistance

5. Chapter 5. Achievements, Final Conclusions and Future Perspectives

Scope of the research

The overall aim of the research presented in this Ph.D. thesis was to establish a robust and reproducible beneficial effect with an elicitor regime on a model and commercially important crop-pathogen system and to investigate the molecular basis to the plant defence response elicited by the treatment regime and the pathogen. The crop and pathogen that fitted this remit were the horticultural edible tomato (*Solanum lycopersicum*) and the fungal necrotrophic pathogen *Botrytis cinerea*, causal agent of grey mould. Chapter 2 presented the findings related to elicitor-long-lasting- (17 dat) and short-duration (4-5 dat)-induced resistance (IR) phenotypical assays against *B. cinerea*, which lead to the identification of a low MW water-soluble chitosan commercialized formulation (ChitoPlant) able to provide long and short-termed IR. This extended to various solanaceous crops of economic or scientific importance, namely *S. lycopersicum*, *S. melongena* and *N. benthamiana*. In all these cases not only was resistance induced but they also showed potential priming-based resistance properties. Also in Chapter 2 the chitosan-enhanced resistance phenotypes were characterised (Figure 5.1) in the mentioned solanaceous crops and *A. thaliana* against *B. cinerea* (Figures 2.4, 2.5, 2.6 and 2.7) and chitosan-priming phase was characterised through a callose deposition assays (Figures 2.16 and 2.17). In the rest of Chapter 2 and the subsequent experimental chapters (Chapters 3 and 4) the molecular basis of chitosan-short-duration-priming (primed and triggered phase, Figure 5.1) were characterised in more depth. In Chapter 3 the mode of action of chitosan as a priming agent is characterised in the tomato transcriptome, demonstrating that chitosan is able to prime

tomato for a more robust and faster gene expression of 2,133 differentially expressed genes (DEGs) whereas non-primed (ddH_2O-treated) and infected tomatoes (Inf) only differentially expressed 363 genes (Figure 3.5). This Chapter also identifies key signalling pathways, such as phytohormones, as necessary for tomato and *A. thaliana* for chitosan-priming for resistance against *B. cinerea*. In Chapter 4 two novel transcripts are identified in two solanaceous crops, tomato and *N. bethamiana*, involved in resistance against *B. cinerea*. Finally, this chapter also reports the potential use of these genes in other plants to enhance resistance against the fungal necrotroph. In this final Chapter 5 the potential applications of these discoveries and the benefits to the horticulture industry are explored.

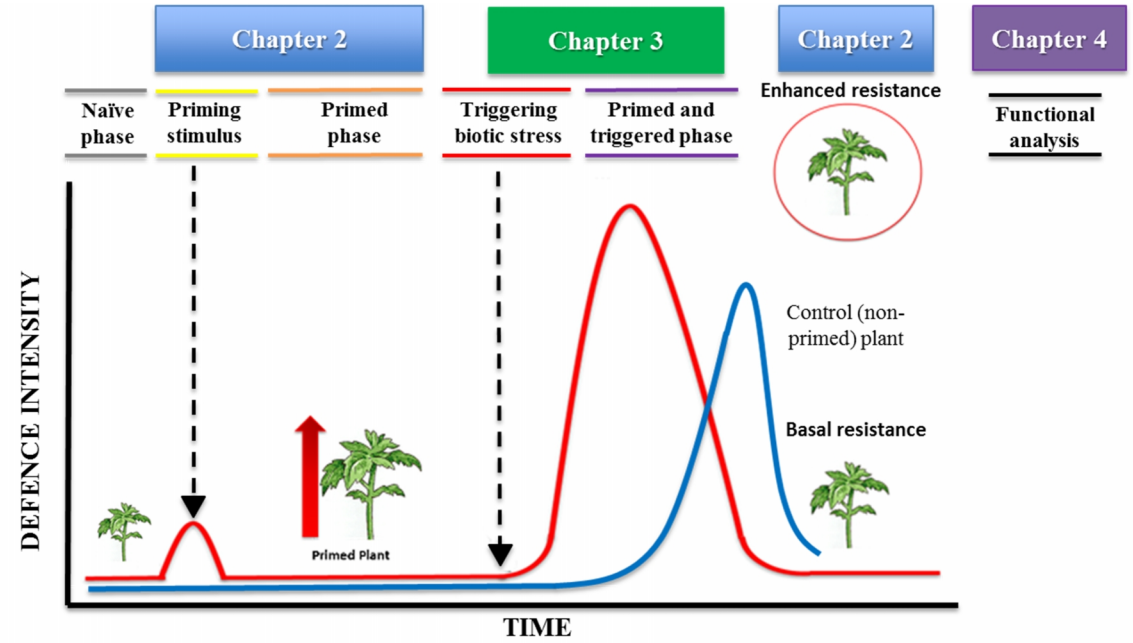


Figure 5.1 Model of a general priming process with an elicitor or priming agent (explained in Chapter 1). Experimental Chapters research fitting within my Ph.D experimental design scope.

Concentration dependence of chitosan as a priming agent and antifungal activity

Chitosan is a well-known PAMP/MAMP but its application as a water-soluble foliar treatment for priming defence and its dose-dependent function as a priming elicitor are novel. This extended to various solanaceous crops and *A. thaliana*. Chitosan antifungal properties have been previously identified (Badawy & Rabea 2014; Romanazzi et al. 2013; Romanazzi et al. 2013; El Hadrami et al. 2010). However, it is important to emphasize that chitosan biological and hence antimicrobial activity depends on the plant model, chemical structure, molecular weight (MW), concentration and its acetylation

degree (DA) (Iriti & Faoro 2009; El Hadrami et al. 2010). In this regard, due to the multifaceted benefits of chitosan in the medical, cosmetic industry and agriculture the extraction efficiency has improved over the years and novel chitosan and chitooligosaccharides formulations have arisen (Cheung et al. 2015; Bueter et al. 2013; Islam & Datta 2015). Nevertheless, to my knowledge few if any studies have investigated chitosan priming properties in crop-pathogen interaction. A chitosan water-soluble formulation with potential priming activity was identified that induced resistance in a dose-related manner in various plants and shown by callose deposition where low-concentration chitosan induced callose strongly and for a longer period than high concentrated. Moreover, high-concentrated chitosan showed strong fungal mycelia and spore germination repression *in vitro* whereas low doses did not have an antifungal effect against *B. cinerea* hypha/spore growth. This discovery clearly demonstrates that chitosan, with a certain MW and DA degree, still depends on its concentration to induce stress (cell death/cytotoxicity) but exhibits clear resistance priming activity once above a minimal threshold concentration and therefore with minimal costs in plant fitness.

Chitosan priming strategy unveiled

The linked transcriptomic and HPLC/MS phytohormone analyses revealed much about how chitosan operates as a priming agent in the tomato immune system against *B. cinerea* during the little studied initial stages of the infection. In general, it was observed that twice as many differentially expressed genes (DEGs) were down-regulated as were up-regulation in both chitosan-primed and triggered plants (Chito+Inf) compared with non-primed and infected plants (Inf) (Figures 3.6, 3.7 and 3.8). Amongst the chitosan-primed and triggered plants where repression was stronger, genes included host susceptible factors that *B. cinerea* requires to promote disease such as glutaredoxin and ethylene receptors (La Camera et al. 2011; Lund et al. 1998), indicating a positive role of chitosan in priming tomato to reduce *B. cinerea* host immune system manipulation. A similar chitosan-related priming effect was observed on fine-tuning hormone-dependent defence gene expression, such as repression of a putative negative regulator of plant defences against necrotrophs, the ABA receptor *SlABAPYL4* (Windram et al. 2012; Asselbergh et al. 2008). These findings open new insights to understand how a priming agent can enhance plant immune systems to counteract necrotrophic pathogen-defence manipulation. This could be validated through reverse genetics to demonstrate whether silencing those genes can enhance

susceptible crop resistance against aggressive necrotrophs. This ultimately could help breeding strategies to identify and target potential susceptibility factors.

Gene ontology (GO) analysis helped to identify that chitosan-priming for tomato resistance against *B. cinerea*, can be divided in five main clusters (Figure 5.2). First, chitosan-primed and triggered plants were able to deploy a more robust signalling network (Appendix) including the stronger expression of receptor-like kinase cascades, calcium-dependent protein kinases, photosynthesis and sugar and nutrient physiology-related genes (Figure 3.13); this indicates a higher cell sensitization and hence an enhanced capacity of defence of the chitosan-primed and triggered cells, as it has been previously associated with priming (Luna et al. 2014; Conrath et al. 2002; Pozo et al. 2004). Second, chitosan had an expected effect on cell-wall related genes; chitosan-primed callose deposition and strong repression of host susceptibility factors such as pectinases and polygalacturonases (PGs); reduced oxidative stress through fine-tuning H_2O_2 and peroxidase (POD) (Figure 2.20) activity and repressing glutaredoxin expression (Figure 3.16), known to benefit *B. cinerea* disease expansion (Choquer et al. 2007; La Camera et al. 2011). Interestingly, some similarities in priming mechanisms have been found with the priming inducer hexanoic acid (Hx), which primes ABA-dependent callose deposition in tomato against *B. cinerea*; and transcriptomic analysis showed that Hx primes tomato antimicrobial genes (e.g. protease inhibitor and endochitinase genes), and fine-tunes redox proccesses at 24 hpi in tomato to stop *B. cinerea* manipulation of host defences (Aranega-Bou et al. 2014; Finiti et al. 2014). However, the present transcriptomic analysis showed chitosan-based stronger and earlier priming mechanism on cell wall-related genes targeting both the host and the pathogen. Third, chitosan-primed cells were able to fine-tune phytohormone-related cross-talk through priming JA, JA-Ile (Figure 3.25) and JA/ET/SA/ABA transcriptional factors (Figure 3.22), therefore reducing *B. cinerea* well-known hormone antagonism manipulation (El Oirdi et al. 2011). Thus, chitosan seems to prime hormone pathways synergism targeting an activation of specific JA/ET-dependent defence against necrotrophs (Boter et al. 2004). A similar pattern has been observed, in a smaller scale, in Hx-primed tomatoes which was able to prime JA and ET biosynthetic pathways, including activation of *ACC*, OPDA and JA-Ile (Finiti et al. 2014; Kravchuk et al. 2011; Vicedo et al. 2009). In contrast, BABA-dependent priming mechanisms in *A. thaliana* depend on the activation of an onset of NPR1-related genes (Kravchuk et al. 2011; Luna

et al. 2014). Interestingly, JA-signalling pathway stimulation from priming agents seems to be uncommon (Kravchuk et al. 2011), making chitosan a great novel priming agent to target host resistance against necrotrophic pathogens. Fourth, secondary metabolism was also primed by chitosan, including the lipid/fatty acid metabolism and phenylpropanoid pathway, known to have an effect on *B. cinerea* (Kliebenstein et al. 2005). Fifth, chitosan-primed tomato cells were able to reduce and/or abolish the expression of *B. cinerea* previously and recently identified virulence factors (Gonzalez-Rodriguez et al. 2014; Rui & Hahn 2007; Leone 1992), such as endo-polygalacturonases (endo-PGs), a hexokinase and uracil phosphoribosyltransferase (Figure 3.23). These insights reveals chitosan priming strategy in tomato against *B. cinerea* and show new opportunities to find novel genes involved in crop resistance against aggressive necrotrophic fungal pathogens, which may facilitate new antifungal strategies and help to include chitosan as a complementary component of integrated crop protection strategies (IPM).

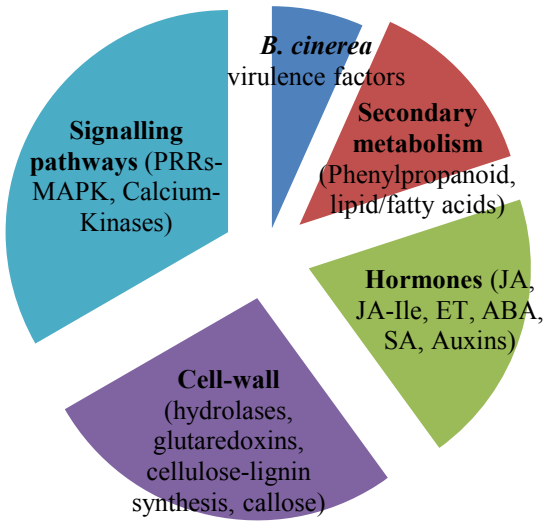


Figure 5.2 Model of chitosan priming and triggering strategy. Five clusters were identified in chitosan-primed and triggered plants (Chito+Inf). These 5 groups include signalling pathways, cell-wall-related genes, phythormone-dependent signalling genes, secondary metabolism and *B. cinerea* virulence factors.

Botrytis cinerea initial stages of the infection

One of the main challenges of this project has been to select a suitable transcriptomic design. As explained before, tomato is a model crop with many –omics analysis studies in biotic/abiotic stresses. Transcriptome analysis design included three specific agents, (i) an elicitor with potential priming properties; (ii) *Solanum lycopersicum*; and (iii) *Botrytis cinerea*. A specific formulation of chitosan, a well-characterised MAMP, was identified for use as the priming agent and at the right priming concentration in this specific pathosystem was determined. The next challenge was to identify the most suitable time points for the infection in order to 'catch' a larger range of 'primed and triggered' (Figures 1.6 and 3.3) sets of transcripts. Another novelty of this transcriptomic analysis was, together with the inclusion of chitosan, the simultaneous analysis of the host and the pathogen transcriptome. Recent studies have revealed that *B. cinerea* can manipulate its host immune system through enhancing the antagonism of hormone signalling pathways (El Oirdi et al. 2011) and its capacity to modulate host redox state. Thus the plant's reactive oxygen species (ROS) production seems to be crucial for the outcome of the infection (Amselem et al. 2011; Asselbergh et al. 2007; Audenaert et al. 2002; Sivakumaran et al. 2016). Indeed, ROS accumulation happens as part of the plant initial defence response after pathogen infection (Mehari et al. 2015). Furthermore, necrotrophic pathogens such as *B. cinerea* are known for their rapid and aggressive infection progress (Figure 3.3), where the majority of transcriptional regulation of the plant genome, including the differential expression of one-third of whole *A. thaliana* genome (Windram et al. 2012), occurs in the first symptomless 48 hpi, right before *B. cinerea* necrotic lesion formation. These insights helped to select the most suitable time points for the transcriptomic analysis.

Identification of *Botrytis cinerea* novel and early-acting virulence factors

An advantage of this transcriptome analysis has been the simultaneous measurement of both the host and the pathogen. However, due to the very early stages of the infection, of the 16,365 *B. cinerea* probes included in the array, only few gene sequences from *B. cinerea* were detected at the 6 hpi and 9 hpi. Nevertheless, novel and well-known virulence factors that *B. cinerea* uses to promote disease were found (Figure 3.23), including superoxide dismutase (SOD), uracil phosphoribosyltransferase (*BcUPRT*) , a hexokinase and various polygalacturonase (PGs) precursor genes, were only found

repressed and/or reduced in their expression by chitosan-primed and infected (Chito+Inf) plants. Interestingly, chitosan at low concentration (0.01 % w/v) was found not to have an antifungal activity on either mycelia or spores (Figure 2.11) indicating an indirect chitosan-related priming effect on the plant. One of *B. cinerea* genes found in the transcriptome to be reduced in chitosan-primed plants was BC1T_12086, a hexokinase, which has been reported to be required for *B. cinerea* sporulation and infection (Rui & Hahn 2007). Furthermore, another chitosan-repressed gene was the enzyme uracil phosphoribosyltransferase, which has been previously identified as a novel virulence factor in *B. cinerea* ungerminated conidia (Gonzalez-Rodriguez et al. 2014). This enzyme is involved in the synthesis of uridine 5'-monophosphate (UMP), a pyrimidine precursor (Villela et al. 2013). Moreover, uracil phosphoribosyltransferase has also been found in *Mycobacterium tuberculosis* (Villela et al. 2013) and *Candida albicans* (Hope et al. 2004) involved in antifungal resistance, indicating a putative common role of this enzyme in pathogenic microbe virulence and antimicrobial resistance. Broad-host range necrotrophs, such as *B. cinerea* (Wang et al. 2014) do not follow a gene-for-gene interaction with the host which makes finding resistance traits even more difficult. The *B. cinerea* transcriptome analysis revealed virulence factors mentioned above which seem to be required for *B. cinerea* sporulation as part of its early secretome, hence pivotal for the initial stages of the infection.

ACRE75 and *ACRE180* role in regulation of plant resistance against *Botrytis cinerea*

An ongoing challenge has been to identify novel genes involved in resistance against *B. cinerea*. Transcriptomic and phytohormone analysis helped to characterise the pathways involved in chitosan-primed and triggered tomato resistance against the fungal necrotroph. Some of these pathways have been previously characterised in *A. thaliana* and tomato, including the role of hormone pathways and plant defence against biotic stresses (Kliebenstein et al. 2005; Ramamoorthy et al. 2002; Dombrecht et al. 2007; Pré et al. 2008; Sivakumaran et al. 2016; Abd El Rahman et al. 2012; El Oirdi et al. 2011). Thus, the challenge was to find genes with unique properties, such as part of a small family of genes to avoid redundancy, for example SR/CAMTA proteins (Li et al. 2014), involved in signalling and with a 'priming expression phenotype' (e.g. being strongly and/or rapidly expressed by Chito+Inf in comparison with Inf plants). Interestingly,

SlACRE75 and *SlACRE180* are two uncharacterised genes with unknown functions, which were found to be primed by chitosan (Figure 4.2) and both being earlier and strongly expressed during the infection. Avr9/Cf-9 rapidly elicited (ACRE) genes were identified by (Durrant et al. 2000) and subsequently characterised (Rowland et al. 2005) where it was found that many of them encode components of signalling cascades, including transcription factors, protein kinases, and ubiquitination pathway-related proteins (González-Lamothe et al. 2006) and hence important for the initial stages of the infection. *ACRE75* and *ACRE180* were demonstrated to be (i) triggered by chitosan and *B. cinerea*; (ii) primed by chitosan; (iii) both co-expressed and share 29 differentially expressed genes (DEGs) including defence-related genes calmodulin/calcium-related genes, exocyst protein, harpin-induced protein, auxins, *BCS1*, WRKYs and matrix metalloproteinase (*Sl2-MMP*); (iv) localized in the nucleus/nucleolus, ER and peroxisomes, do not have signal peptide and therefore presumably involved in intracellular cytoplasmic defence signalling; and (v) positive regulators in plant resistance against *B. cinerea*.

Applications, concluding remarks and future perspectives

Plant immune system is a complex phenomenon that can counteract numerous microbes and would-be pathogens infection. This Ph.D thesis has focused on induced resistance in tomato with the use of elicitors as an alternative or complementary treatment to reduce the use of fungicides in the UK horticultural industry. Research on the different phases of priming (Figure 5.1) as a potential mechanism to help achieve this objective has increased the knowledge of elicitor mode of action in the crop and in priming plant defences against fungal attack, which can ultimately provide the tools to continue to achieve effective crop protection that might otherwise be compromised by loss of crop protection products or their reduced efficacy.

The nature of the research reported is to investigate common mechanisms of plant defence and therefore has wide potential application compared with, for example, a focus on fungicides limited to a single group of crops. Therefore, the work can be seen as under-pinning crop protection mechanisms, establishing principles and potential for using resistance elicitors in robust integrated crop protection strategies. This breadth of application potential is demonstrated by chitosan-induced resistance phenotype achieved in two tomato cultivars, in various solanaceous crops and in a plant belonging

to the *Brassicaceae* family, *A.thaliana*, which indicates a common perception mechanism and hence similar protection levels of chitosan among plant species and even families. Future research should aim to optimise an elicitor regime that includes chitosan in various crops and to study its protection level against different pathogens. The use of chitosan in combination with other elicitors such as MeJA showed the potential for stronger protection levels without compromising or over-stressing plant hormonal synthesis (Figures 3.24, 3.25 and 3.26). These findings clearly show the potential synergistic effects of chitosan in a dose-dependent manner, and hence to include chitosan and MeJA as complementary components in crop protection protocols as a defence inducer or priming agent against fungal pathogens. Thus, these findings may help to determine the principles whereby such products can be used, and particularly how they might be co mbined effectively. The latter will be as much about avoiding detrimental combination and practices as identifying those that might be additive or synergistic.

Plant cultivars have been domesticated over hundreds of years for improve yield and fruit quality and grown in low-stress facilities, such as greenhouses and nurseries which has likely contributed to a decreased in disease resistance over their wild-type ancestors (Burketova et al. 2015). However, it was demonstrated even susceptible tomato cultivars can be primed with chitosan for a more robust and fine-tuned defence response, when triggered in a focussed, specifically-targeted way, which ultimately can prevent disease and reduce the need for conventional fungicide use.

It was demonstrated that two tomato genes, *SlACRE75* and *SlACRE180,* and their *N. benthamiana* orthologues, reported to be implicated in resistance to biotroph tomato leaf mould (*Cladosporium fulvum*) and involved in plant defences against biotic stress (Wendy E Durrant et al. 2000; Rowland et al. 2005), also have role in resistance against the necrotroph pathogen *B. cinerea*. Transcriptomic and qRT-PCR analysis showed that chitosan can prime the four genes for a faster and/or stronger expression. Similar ACRE genes are also present in other solanaceous crops, such as potato and tobacco, suggesting that the same chitosan-induced resistance mechanism might also be exploited in other plant systems. Introducing and/or priming *SlACRE75*, *SlACRE180*, *NbACRE75* and /or *NbACRE180* into other crop families (e.g. Brassicaceae), by classical crop breeding or through molecular means, might enhance the tolerance and/or

resistance of domestic susceptible varieties to old and emergent pest and pathogens, and other stresses.

These results unveiled potential molecular pathways involved in chitosan-induced priming for resistance in tomato, and potentially applicable to other crops against *B. cinerea*. This information could help breeders to target germplasm with a stronger expression of key pathways identified in order to develop cultivars more resistant against aggressive fungal pathogens.

Finally, the discovery that uracil phosphoribosyltransferase (*BcUPRT*), a novel *B. cinerea* virulence factor involved in spore germination was repressed in chitosan-primed tomatoes was a potentially important finding (Figure 3.23). Future research should aim to understand the importance of this candidate for the pathogen and its infection. This could be done by (i) transiently silence the pathogen gene *in planta*, creating plants producing *B. cinerea* uracil phosphoribosyltransferase (*BcUPRT*) antisense gene with Host-Induced Gene Silencing (HIGS) (Nowara et al. 2010), or (ii) creating a constitutive mutant (expressing 35S: *BcUPRT* antisense) that would potentially inhibit *BcUPRT* expression upon *B. cinerea* initial infection, however to my knowledge targeting a fungal gene *in planta* has not been done before. These mutants would be investigated to look for whether they potentially stop and/or reduce *B. cinerea* sporulation/mycelia production and hence disease expansion. However, silencing of fungal genes *in planta* may be limited by the plant RNA interaction with fungal RNA.

An alternative to gene silencing could be to transiently or constitutively overexpress (OE) *BcUPRT* in the plant to determine whether the plant is affected by the protein. Moreover, an infection/pathogenicity assay could be performed with *B. cinerea* to investigate whether the OE transformants are more susceptible and trigger faster fungal growth/lesion expansion. If the importance for pathogeniciy of this candidate is proven, these results open new possibilities to include chitosan and to study *BcUPRT* enzyme as a potential target for the development of new fungicides (Gonzalez-Rodriguez et al. 2014).

APPENDIX

Gateway-compatible entry and destination vectors

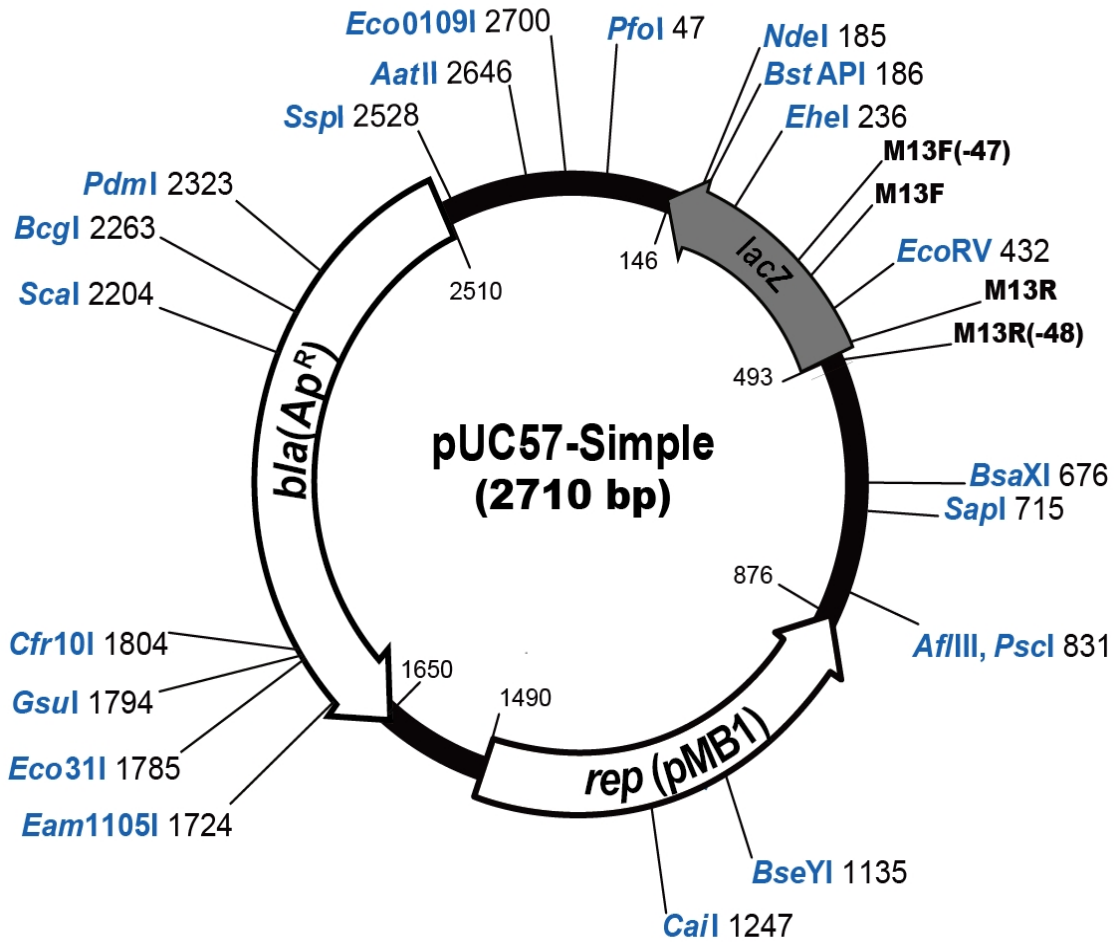


Figure A.1 pUC57 (entry vector)

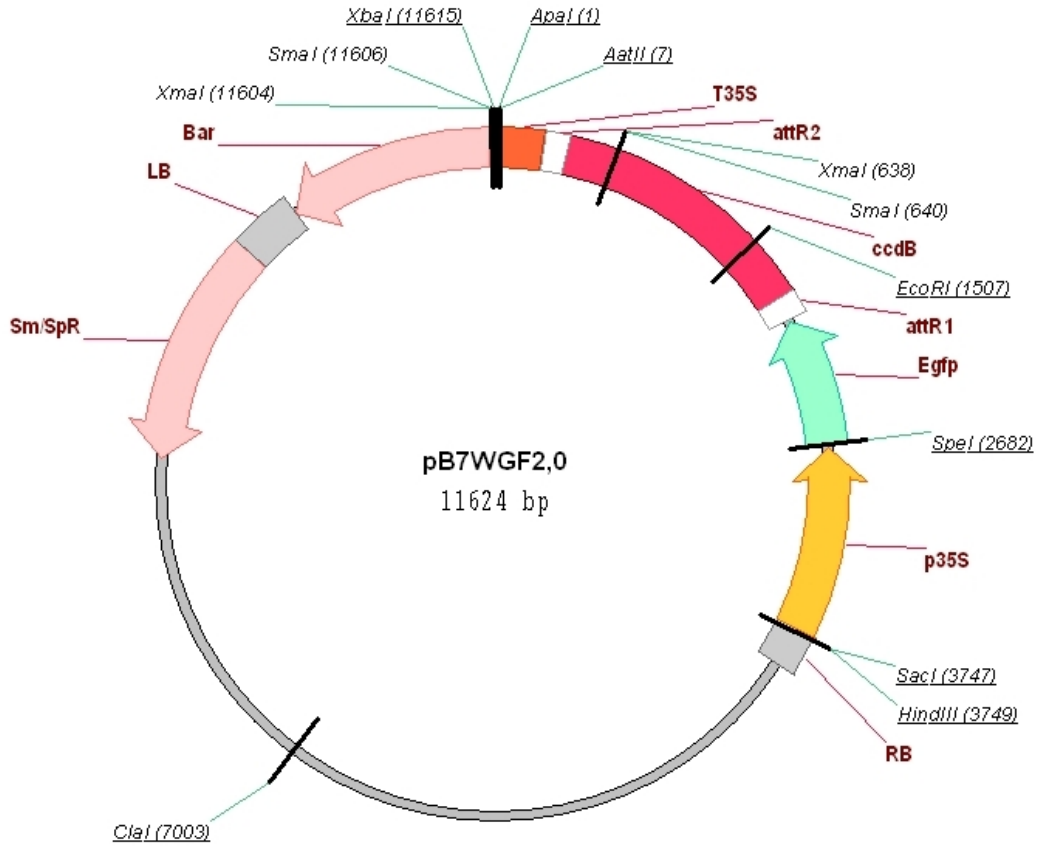


Figure A.2 Destination vector (pB7WGF2) (Karimi et al. 2002)

Gene constructs sequences with expected product sizes

1. SlACRE75 product size: 679 bp (M13Fwd-attl1-mygene-attl2-M13Reverse)

Tgtaaaacgacggccagtgaattcaaataatgattttattttgactgatagtgacctgttcgttgcaacaaattgatgagcaatgct
tttttataatgccaactttgtacaaaaaagcaggcatggcattttcggagccaatagttatatgcaagaagcatccacaacacaaa
caacaacccggagtatgttcatgttgtttaagagaaaagttatcaaagatcaatggtacaactacaatagctaaaatttcatctatt
ccttcttctatatcctcctccgtttcctcgtccaactgcaactctccacgagctgcaattggacatcgtcgaatcacgtccgatgttg
ctggcggccactattcgttcgtagcgctagcggccgccaacgctggaggaggaggaggggggttgaagaagagtaggtca
atagcttttgttgctagaggggtagagttggaattaatggaaaaaagaagaaagaagggttttggtcaaagttgatccggtcaa
cggggaaacggaccaatcgggttgttgtgcattgacagctttcttgtacaaagttggcattataagaaagcattgcttatcaatttg
ttgcaacgaacaggtcactatcagtcaaaataaaatcattatttaagcttggcgtaatcatggtcatagctgtttcctg

2. SlACRE180 product size: 613bp (M13Fwd-attl1-mygene-attl2-M13Reverse)

tgtaaaacgacggccagtgaattcgagctcggtacctcgcgaatgcatctagatgaattcaaataatgattttattttgactgatag
tgacctgttcgttgcaacaaattgatgagcaatgctttttataatgccaactttgtacaaaaaagcaggcatgaaagagagcggt
tctttgttgtcattcaagaatagaaatggcggtgattctcatgatgacgatgataataataataattctaggacttataaggcaa
cctcattgagacggagatttaggaatttgtgttcgcggaagaagaagagtctatacaaattgtgtcatcatcggttcgttgaattta
ttttattcaagattgcctctcttttggaagcaattgctatggttgctaccttagttttcttcttcttccgttatggttttcatatttgacagctt
tcttgtacaaagttggcattataagaaagcattgcttatcaatttgttgcaacgaacaggtcactatcagtcaaaataaaatcattatt
taagcttatcggatcccgggcccgtcgactgcagaggcctgcatgcaagcttggcgtaatcatggtcatagctgtttcctg

3. NbenthACRE180 product size: 643bp (M13Fwd-attl1-mygene-attl2-M13Reverse)

Tgtaaaacgacggccagtgaattcgagctcggtacctcgcgaatgcatctagatgaattcaaataatgattttattttgactgata
gtgacctgttcgttgcaacaaattgatgagcaatgctttttataatgccaactttgtacaaaaaagcaggcatgaaagcgggtgg
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atacaaattgcatcattggtttgttgattcctttctattcaagattgtttctgtttttgaagccattgctttggtttccaccttagctttcttct
tcttttgttgtggttgccatatctgacagctttcttgtacaaagttggcattataagaaagcattgcttatcaatttgttgcaacgaaca
ggtcactatcagtcaaaataaaatcattatttaagcttatcggatcccgggcccgtcgactgcagaggcctgcatgcaagcttgg
cgtaatcatggtcatagctgtttcctg

4.NbenthACRE75 product size: 739bp (M13Fwd-attl1-mygene-attl2-M13Reverse)

tgtaaaacgacggccagtgaattcaaataatgattttattttgactgatagtgacctgttcgttgcaacaaattgatgagcaatgctt
ttttataatgccaactttgtacaaaaaagcaggcatggctttttcagaaccagaagtaatatgcaagaagcacccacaacacaaa
caacaaccaggagtatgttcttgttgtctaagagaaaagctctccaaaattaatggtacatctacaatactggatgcccttgcttca
atttcttcttctttatcttcctctactacttcatccaattgcgtctcgccacggggagccatcatccaccgtcggatcacctccgacgt
caccggcggccattactcctttgtcagtctcgccggcggaggcaccggcggtggtttgaagaagagtagatcaattgctattgt
tgctagaggtggtagagttggaattaatggaaagaagaagaaagaaggtttttggtcaaagttgatcaagtcaacggggaaga
ggactaaaagggtaattgcacattgacagctttcttgtacaaagttggcattataagaaagcattgcttatcaatttgttgcaacga
acaggtcactatcagtcaaaataaaatcattatttaagcttggcgtaatcatggtcatagctatcggatcccgggcccgtcgactg
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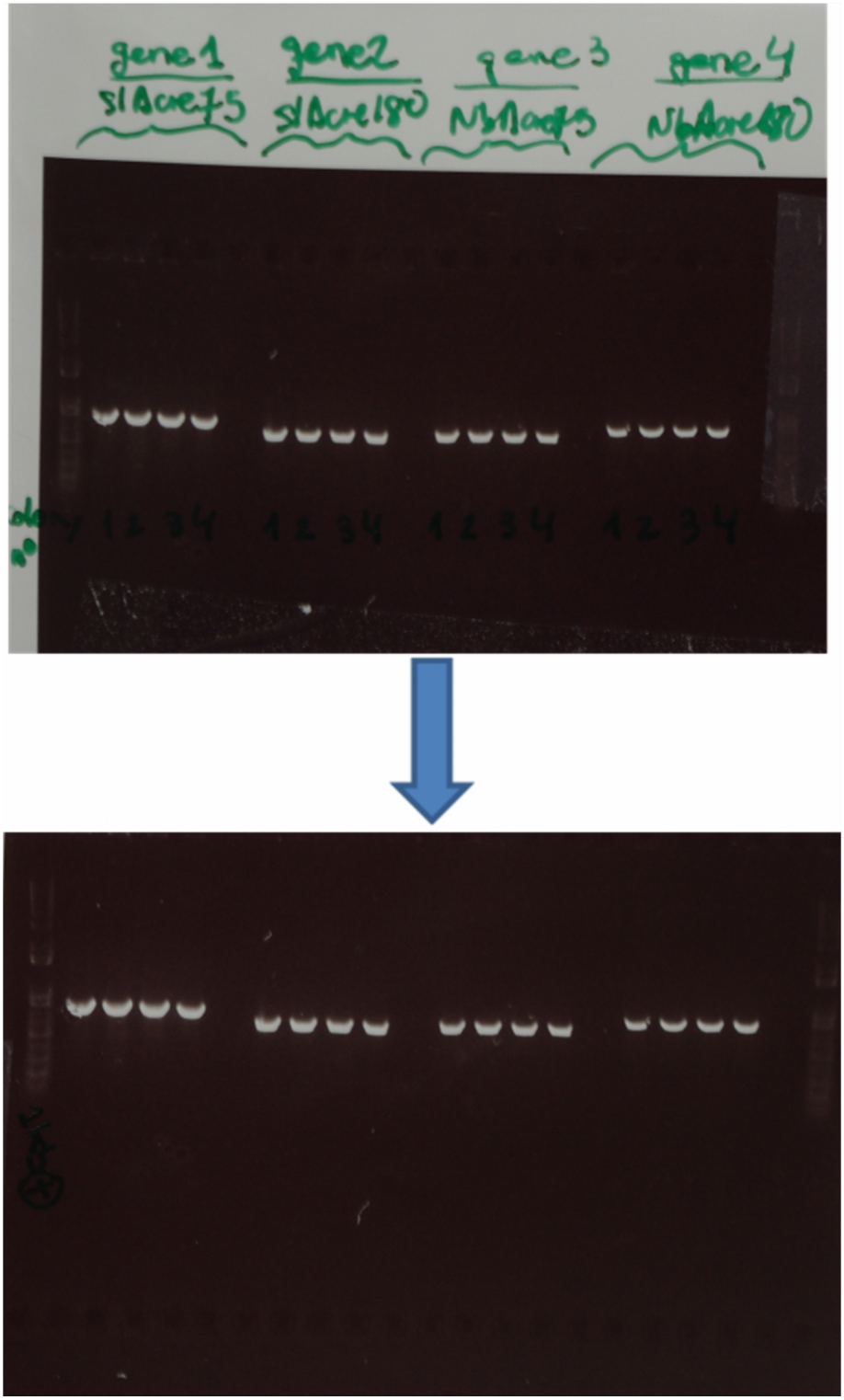


Figure A.3. Colony PCR pUC57 + gene inserts. *SlACRE75*, *SlACRE180*, *NbACRE180* and *NbACRE75*.

Western Blot for *SlACRE75*, *SlACRE180*, *NbACRE75* and *NbACRE180* proteins

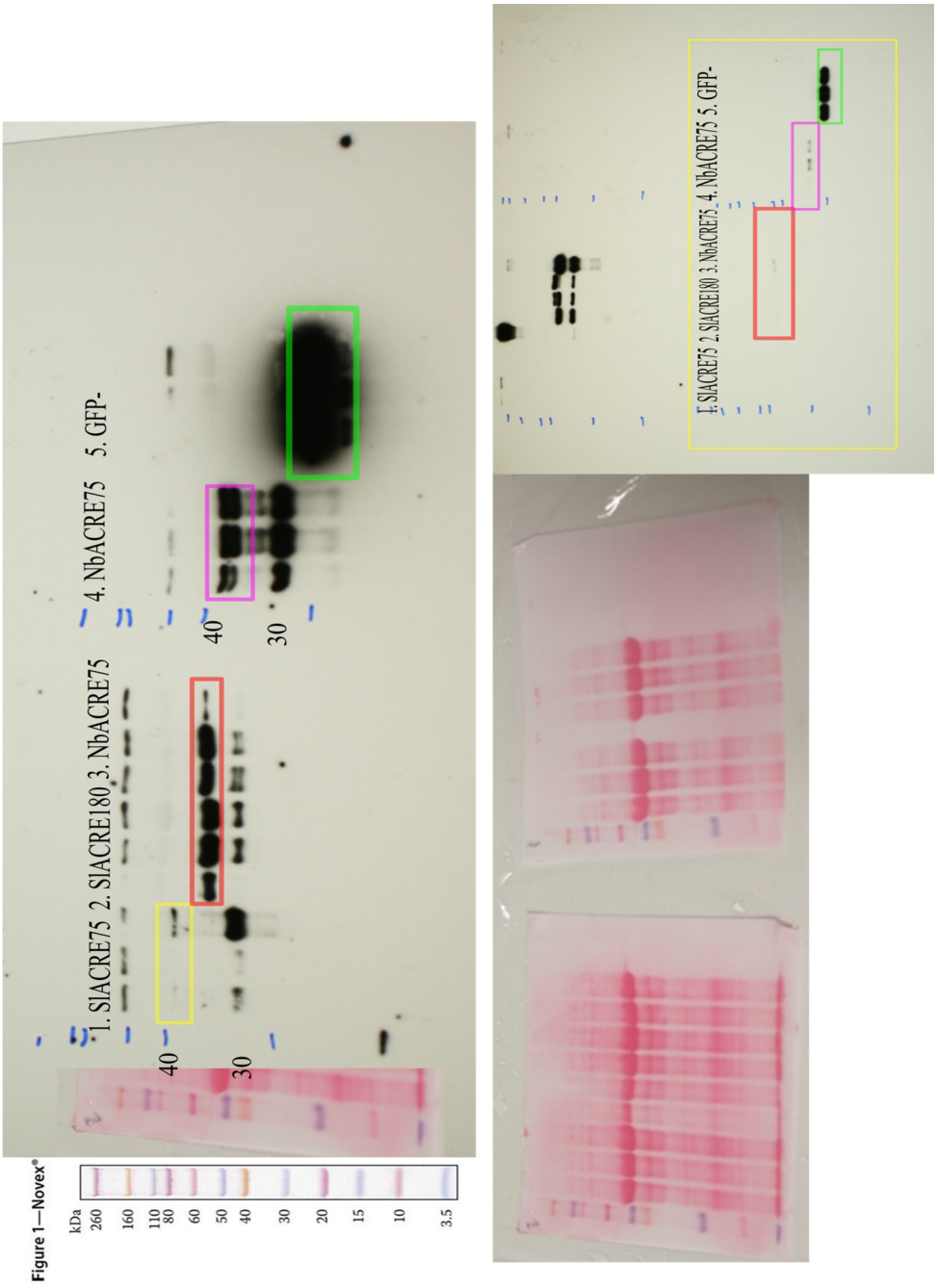


Figure A.3 Expression of proteins by immunoblot analysis of GFP-SlACRE75, GFP-SlACRE180, GFP-NbACRE75 and GFP-NbACRE180 fusion proteins in N. benthamiana leaves at 48 h after agroinfiltration. Expected protein sizes were (i) SlACRE75= 14.79 + 26 KDa GFP= 40.8 KDa; (ii) SlACRE180= 10.86 +26= 36.8 KDa; (iii) NbACRE180= 11.74+26= 37.7 KDa; and (iv) NbACRE75= 14.6+26= 40.7 KDa

Signal peptides predictions for *SlACRE75*, *SlACRE180*, *NbACRE75* and *NbACRE180* proteins

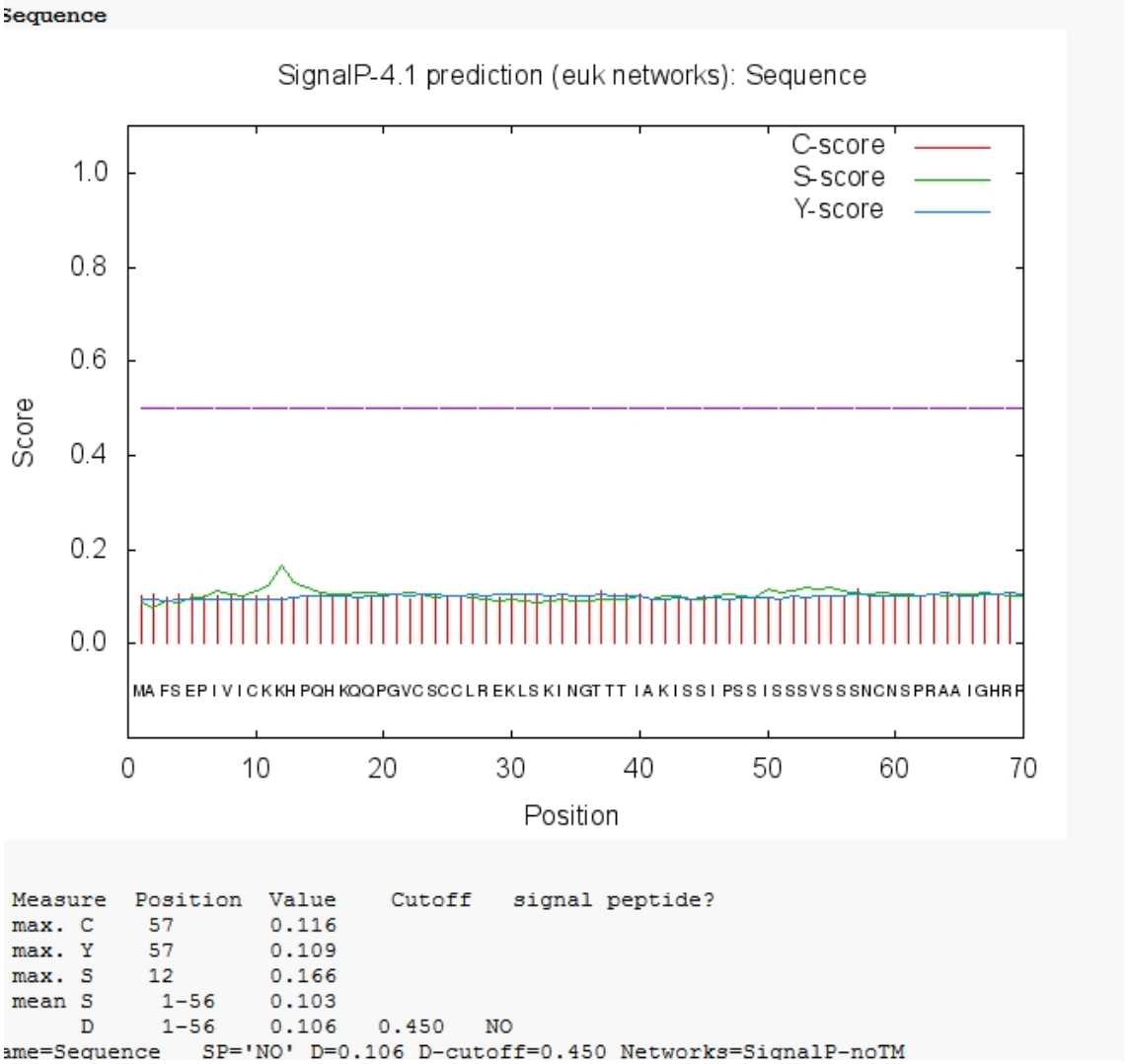
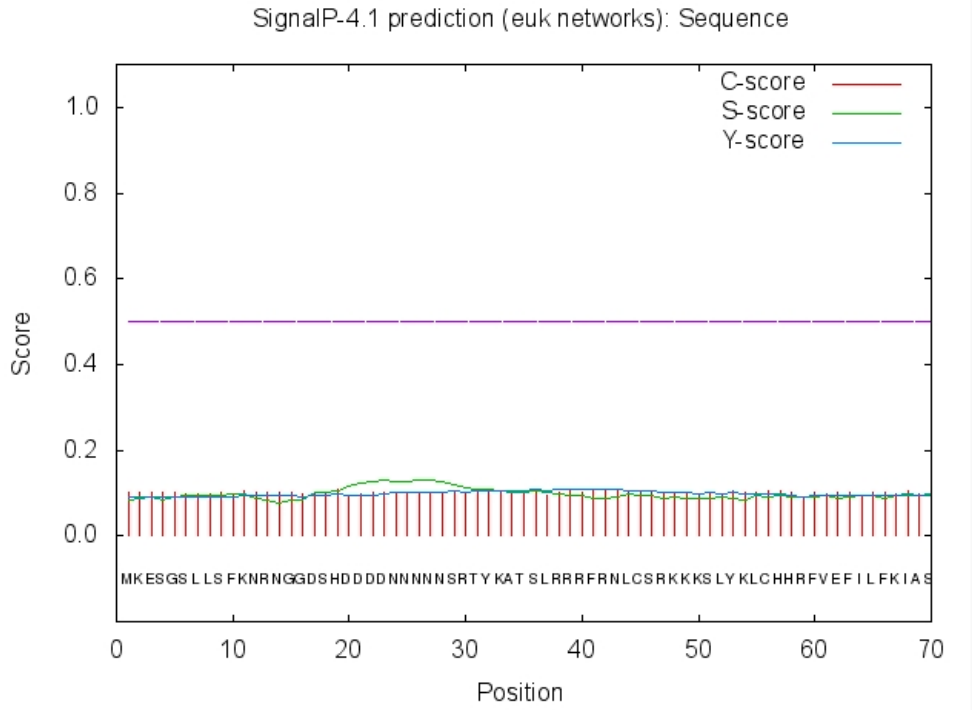


Figure A.4 *SlACRE75* presence and location of signal peptide cleavage site prediction by SignalP 4.1 Server (http://www.cbs.dtu. dk/services/SignalP).

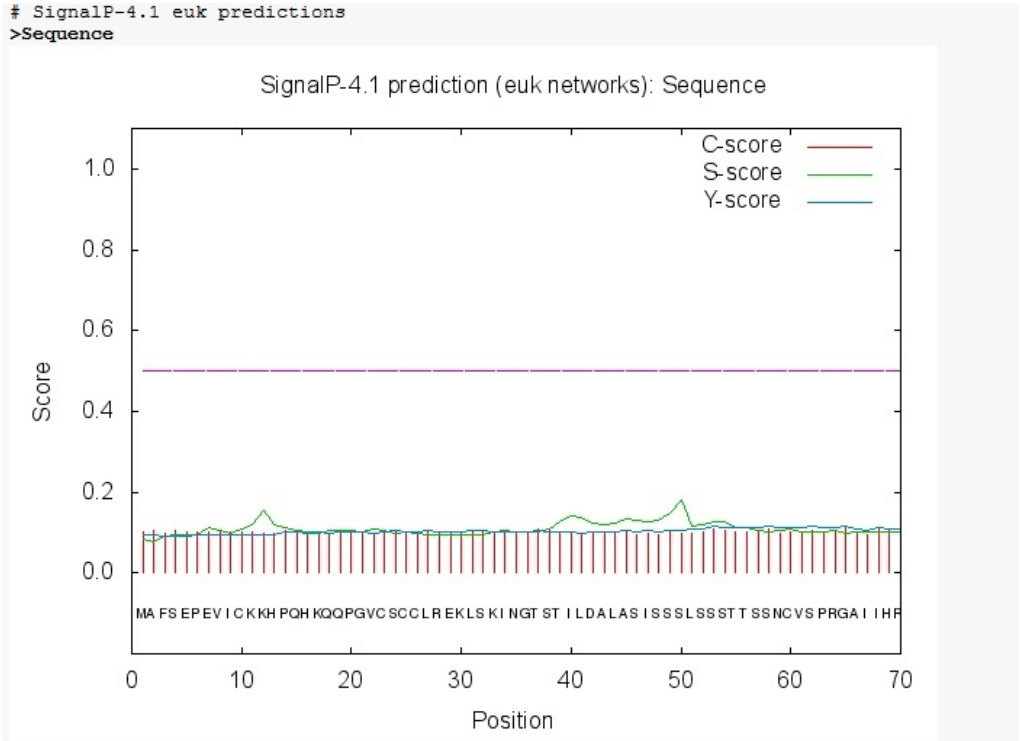
SignalP-4.1 euk predictions
Sequence



Measure	Position	Value	Cutoff	signal peptide?
max. C	19	0.110		
max. Y	42	0.110		
max. S	23	0.131		
mean S	1-41	0.103		
D	1-41	0.106	0.450	NO

ame=Sequence SP='NO' D=0.106 D-cutoff=0.450 Networks=SignalP-noTM

Figure A.5 *SlACRE180* presence and location of signal peptide cleavage site prediction by SignalP 4.1 Server (http://www.cbs.dtu. dk/services/SignalP).

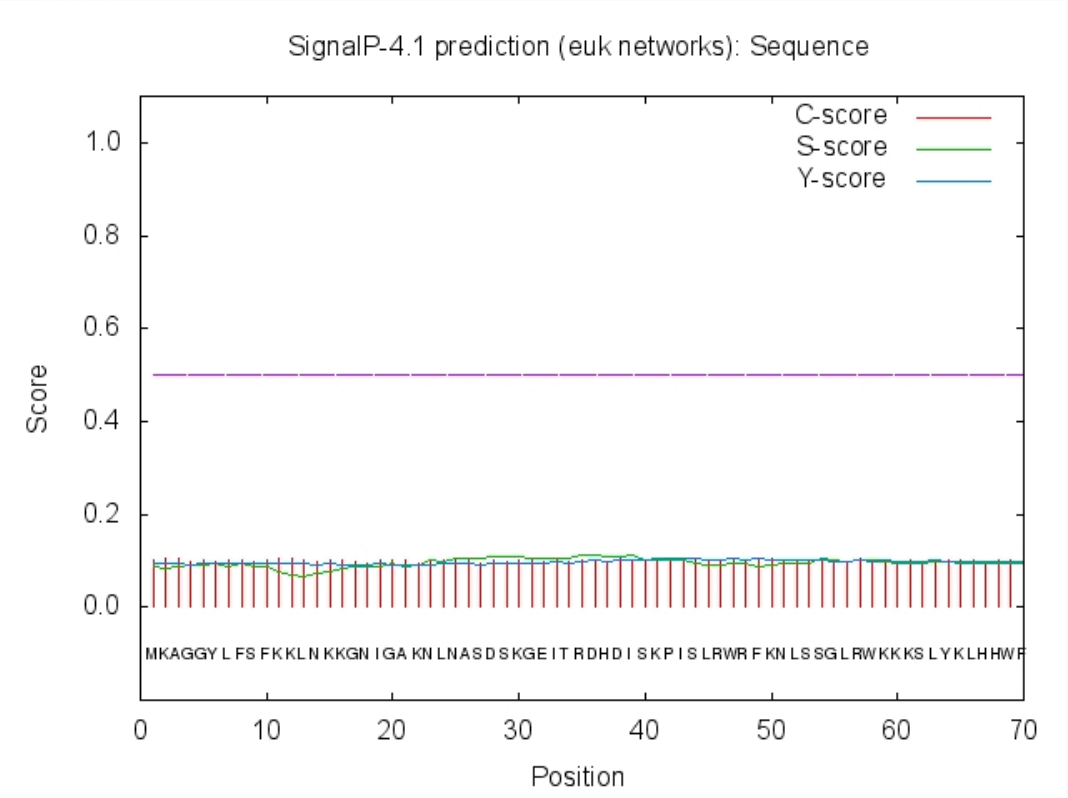



```
# SignalP-4.1 euk predictions
>Sequence

# Measure  Position  Value    Cutoff   signal peptide?
  max. C    65       0.111
  max. Y    58       0.117
  max. S    50       0.182
  mean S     1-57    0.111
       D     1-57    0.114   0.450   NO
Name=Sequence   SP='NO' D=0.114 D-cutoff=0.450 Networks=SignalP-noTM
```

Figure A.6. *NbACRE75* presence and location of signal peptide cleavage site prediction by SignalP 4.1 Server (http://www.cbs.dtu. dk/services/SignalP).

```
# SignalP-4.1 euk predictions
>Sequence
```




```
# Measure  Position  Value    Cutoff   signal peptide?
  max. C    43        0.107
  max. Y    43        0.106
  max. S    39        0.111
  mean S     1-42     0.095
       D     1-42     0.100   0.450   NO
Name=Sequence   SP='NO' D=0.100 D-cutoff=0.450 Networks=SignalP-noTM
```

Figure A.7. *NbACRE180* presence and location of signal peptide cleavage site prediction by SignalP 4.1 Server (http://www.cbs.dtu. dk/services/SignalP).

Singular enrichment analysis (SEA) with the AgriGO for Inf treatment at 6, 9 and 12 hpi

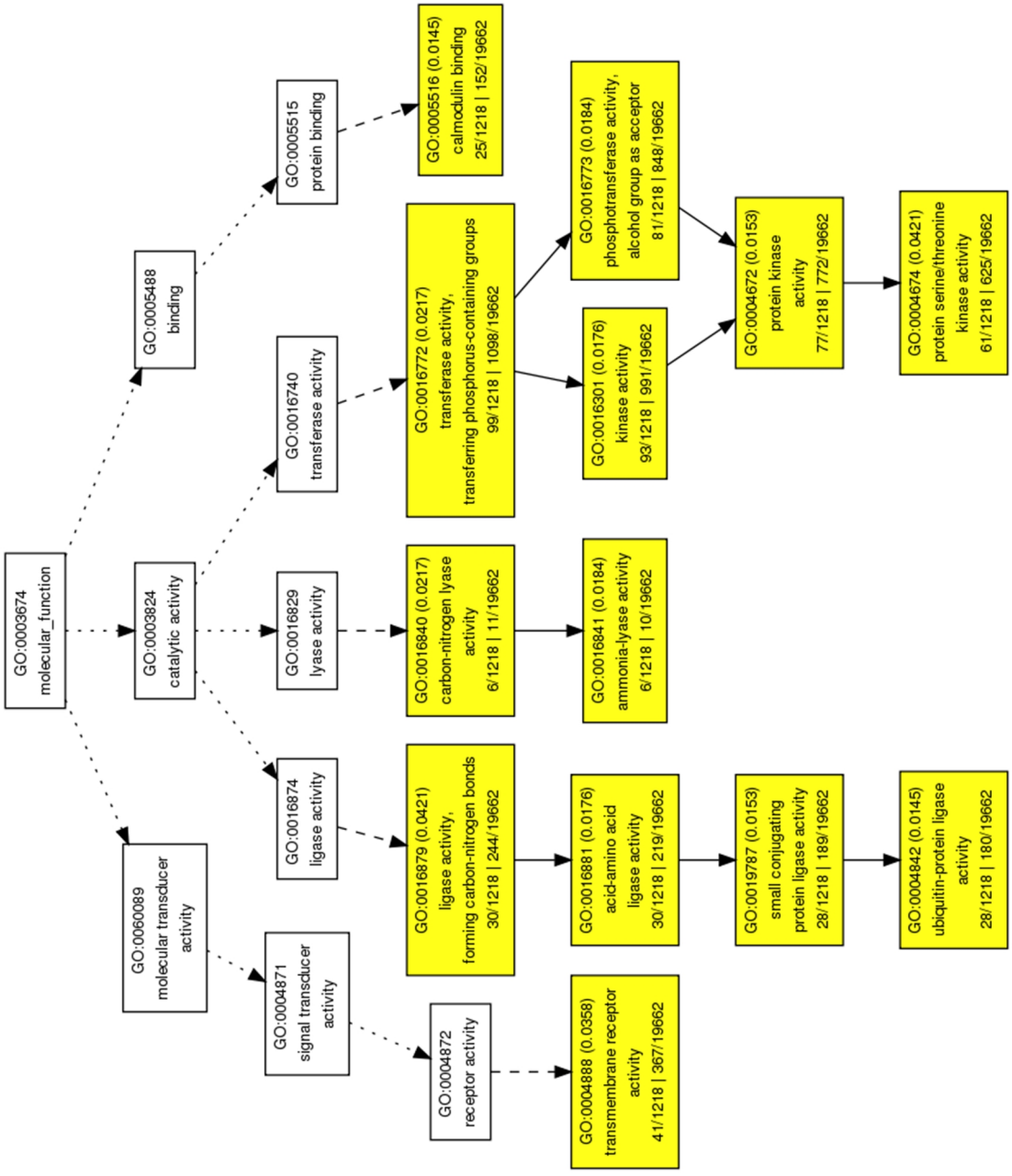


Figure A.8. Functional enrichment of non-primed (ddH_2O-treated) and *B. cinerea*-infected (Inf) differentially expressed genes (DEGs) was analysed by singular enrichment analysis (SEA) with the AgriGO tool.

Singular enrichment analysis (SEA) with the AgriGO for Chito+Inf treatment at 6, 9 and 12 hpi

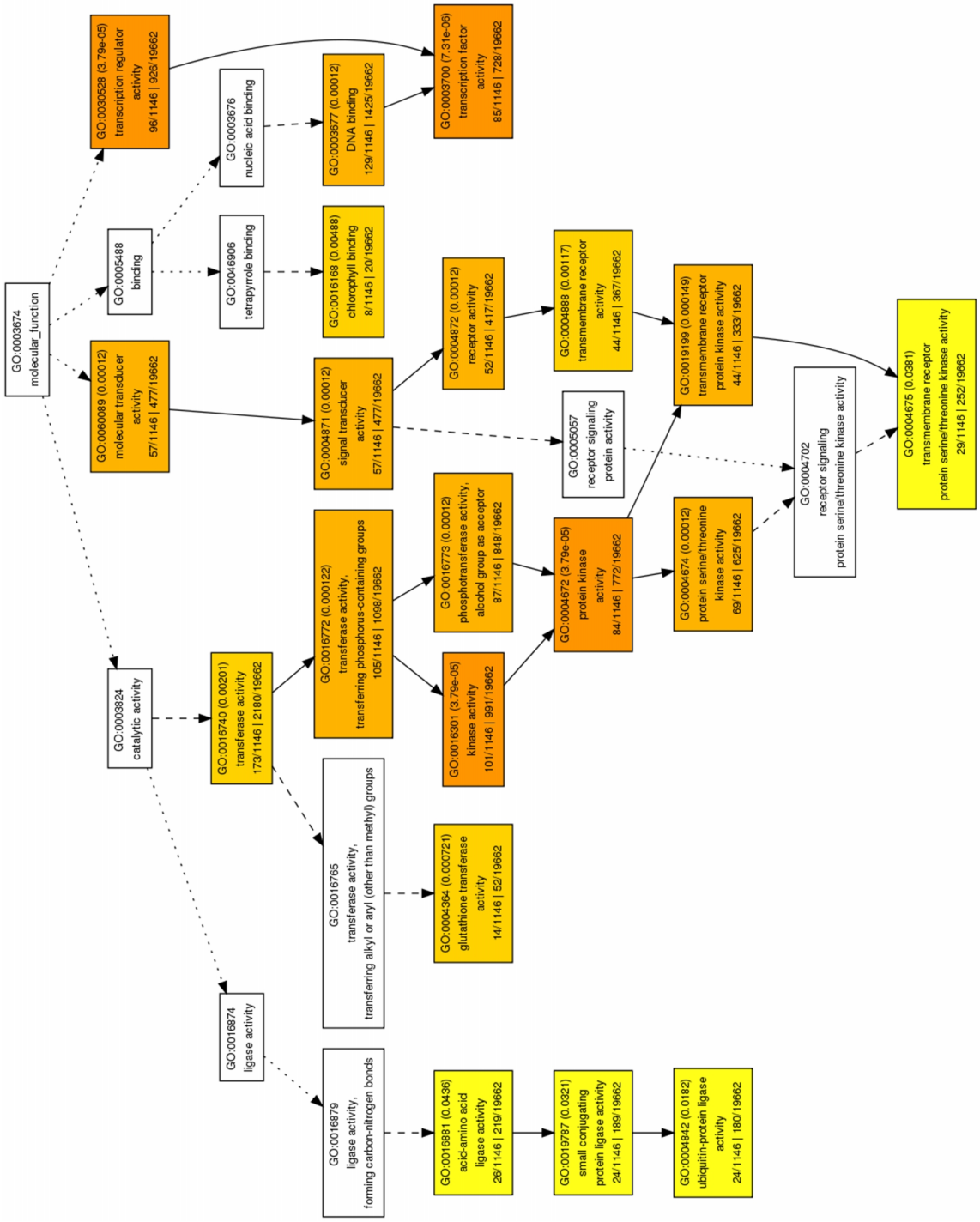


Figure A.9. Functional enrichment of chitosan-primed and *B. cinerea*-infected (Chito+Inf) differentially expressed genes (DEGs) was analysed by singular enrichment analysis (SEA) with the AgriGO tool.

Oligonucleotides primers

Primer name	Primer sequence 5' - 3'	UPL Probe	Purpose
SlActinF	TCCCTCGTAAATTGGGACAG	9	qRT-PCR reference gene
SlActinR	TACCACCGGTATTGTGTTGG		
SlUbiquitinF	TCTCCGTGGTGGTGCTAAG	143	qRT-PCR reference gene
SlUbiquitinR	TTGATCTTCTTTGGCTTGGTG		
SlACRE75F	GGCCACTATTCGTTCGTAGC	65	qRT-PCR
SlACRE75R	AGCTATTGACCTACTCTTCTTCAACC		
SlACRE180F	TGAGACGGAGATTTAGGAATTTG	143	qRT-PCR
SlACRE180R	CAACGAACCGATGATGACAC		
SlPOD5F	TGCTGTAATGTTGGTTAATCCTTC	133	qRT-PCR
SlPOD5R	CAAACCAGGCTGGAGCTAAT		
SlCHI2F	TGTGTCTCAATTCATGGACTATTTTT	134	qRT-PCR
SlCHI2R	CCGAAGCTAGCCAAGATCC		
SlEGaseF	TCTGCACCGATTCATTTGAC	138	qRT-PCR
SlEGaseR	ACACCTGAGAACCCACCAGA		
SlLBD42F	CGGTTTAAACGGGTATCAGC	38	qRT-PCR
SlLBD42R	TAGCCGCATCGTCAACATC		
SlPtoSer/threKFwd	CTTGGTCTTTTCTCCAAGATTGA	73	qRT-PCR
SlPtoSer/threKRev	AGGCAGCCAGACATCTCAAG		
SlRKaseF	AAGTCCTACTGATACTTGCTACCTCA	7	qRT-PCR
SlRKaseF	TCAAGGTCTTGAAAGAATCGAAG		
SlGSTFwd	TCGACATCGTTGCCAGTTAC	126	qRT-PCR
SlGSTRev	CAACAGTTCCATCCCTATTGC		
NbActinF	TCTATAACGAGCTTCGTGTTGC	82	qRT-PCR reference gene
NbActinR	GAGGTGCTTCAGTTAGGAGGAC		
NbEF1alphaF	ATTGAGAAGGAGCCCAAGTTC	9	qRT-PCR reference gene
NbEF1alphaR	GGGAAGCATCCTAACCATACC		
NbUbiquitinF	ACTTACACCAAGCCTAAGAAGATTAAG	143	qRT-PCR reference
NbUbiquitinR	TCCACCTTGTAGAACTGAAGCAC		

			gene
NbACRE75F	ACAATACTGGATGCCCTTGC	143	qRT-PCR
NbACRE75R	CGAGACGCAATTGGATGA		
NbACRE180F	CTAAAGGAGAAATAACAAGGGATCA	39	qRT-PCR
NbACRE180R	TTTTTCTTCCACCTTAAACCAGA		
NbACRE31F	AATTCGGCCATCGTGATCTTGGTC	-	qRT-PCR
NbACRE31R	GAGAAACTGGGATTGCCTGAAGGA		
NbPTI5F	CCTCCAAGTTTGAGCTCGGATAGT	-	qRT-PCR
NbPTI5R	CCAAGAAATTCTCCATGCACTCTGTC		
NbWRKY7F	CACAAGGGTACAAACAACACAG	-	qRT-PCR
NbWRKY7R	GGTTGCATTTGGTTCATGTAAG		
NbWRKY8F	AACAATGGTGCCAATAATGC	-	qRT-PCR
NbWRKY8R	TGCATATCCTGAGAAACCATT		
M13Fwd	GTAAAACGACGGCCAGT		Colony PCR
M13Rev	GGAAACAGCTATGACCATG		
SlLapAFwd	ATCTCAGGTTTCCTGGTGGAAGGA		qRT-PCR
SlLapARev	AGTTGCTATGGCAGAGGCAGAG		
SlLoxDFwd	CCGTGGTTGACACATTATCG		qRT-PCR
SlLoxDRev	ACAGCAGTCCGCCCTATTTA		
SlEF1αFwd	CTCCGTCTTCCACTTCAGG		qRT-PCR
SlEF1αRev	TCAGTTGTCAAACCAGTAGGG		
SlNPR1Fwd	GGGAAAGATAGCAGCACG		qRT-PCR
SlNPR1Rev	GTCCACACAAACACACACATC		
SlActinFwd	GCAGGACGTGACCTCA		qRT-PCR
SlActinRev	GGAAGGTGCTGAGGGAA		
SlPR-1Fwd	TGTCCGAGAGGCCAAGCTATAAC		qRT-PCR
SlPR-1Rev	AATGAACCACCATCCGTTGTTGC		
SlPin1 (PI-I)Fwd	GAAACTCTCATGGCACGAA		qRT-PCR
SlPin1 (PI-I)Rev	CCTTCGCACATCAAGTTAGAG		

Table A.1 Oligonucleotide primer list

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